Different Effect of the Ca$^{2+}$ Sensitizers EMD 57033 and CGP 48506 on Cross-Bridge Cycling in Human Myocardium

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ABSTRACT

Ca$^{2+}$ sensitizers may be advantageous for treatment in human heart failure by increasing cardiac force without increasing the Ca$^{2+}$ transient or energy consumption. To study the mode of action of the Ca$^{2+}$ sensitizers EMD 57033 (EMD) and CGP 48506 (CGP), their influence on butanedione monoxide (BDM)-mediated depression of cross-bridge cycling was analyzed in human myocardium (explanted hearts, dilated cardiomyopathy, n = 19). In Triton X (1%)-skinned fiber preparations of left ventricular myocardium from patients suffering from dilated cardiomyopathy, troponin I was extracted by vanadate (10 mM) treatment, resulting in a Ca$^{2+}$-independent contraction. In troponin I-depleted fibers BDM (5–50 mM) was applied in the absence and presence of EMD (10 μM) or CGP (10 μM). To analyze the influence on cross-bridge kinetics, tension cost (ratio of ATPase activity and tension development) was studied.

BDM exerted a dose-dependent force inhibition in troponin I-depleted fibers (IC$_{50}$ = 7.22 mM), which was antagonized by EMD (IC$_{50}$ of BDM + EMD = 19.97 mM) and CGP (IC$_{50}$ of BDM + CGP = 15.30 mM). EMD increased Ca$^{2+}$ sensitivity of force and maximal force in Triton X-skinned fibers. The Ca$^{2+}$-sensitizing effect of CGP was accompanied by an increased Ca$^{2+}$ sensitivity of myosin-ATPase activity, an increased slope of the Ca$^{2+}$ force and Ca$^{2+}$ ATPase curve, as well as a reduced maximal myosin ATPase activity. CGP and EMD reduced tension cost. In conclusion, EMD and CGP antagonize the BDM-mediated relaxation in troponin I-depleted cardiac muscle fibers. The Ca$^{2+}$-sensitizing effect of CGP seems to be dependent on an improvement of the myofilament cooperativity, whereas EMD seems to operate by increasing the force per cross-bridge.

2,3-Butanediol monoxide (BDM) has been characterized as a nucleophile oXime with a phosphatase-like activity (Coulombe et al., 1990). On the level of the contractile apparatus, BDM exerts a Ca$^{2+}$-desensitizing effect on cardiac as well as on skeletal skinned muscle fibers (Fryer et al., 1988; Gwathmey et al., 1991). Accordingly, BDM has been shown to inhibit the actomyosin ATPase (Higuchi and Takemori, 1989; McKillop et al., 1994). From experiments demonstrating that BDM decreases contractile force of skinned fibers at maximal calcium activation, but less than immediate stiffness, it was concluded that BDM increases the population of strongly attached “preforce-generating” cross-bridges, possibly by slowing and inhibiting the inorganic phosphate (P$_i$) release step and stabilizing the actomyosin-ADP-P$_i$ intermediate cross-bridge state (actomyosin-ADP-P$_i$) (Zhao and Kawai, 1994). Experiments on frog muscle have shown that BDM decreases the rate of cross-bridge attachment and the force per cross-bridge (Bagni et al., 1992). From simultaneous measurements of force and myosin ATPase activity in skinned cardiac rat trabeculae, it was concluded that BDM not only affects cross-bridge formation but also causes an increase in the apparent rate of cross-bridge detachment (Ebus and Stienen, 1996).

The thiadiazinone derivative EMD 53998 (EMD, 5-[1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one) and its (+)-enantiomer EMD 57033 are classified as Ca$^{2+}$ sensitizers (Beier et al., 1991; Solaro et al., 1993). Both compounds seem to directly interact with the actomyosin contractile system (Strauss et al., 1992a; Solaro et al., 1993). EMD 53998 increases the rate of cross-bridge attachment in the strongly bound force-generating state (Simnett et al., 1993a; Arner et al., 1995), whereas the cross-bridge detachment rate (g$_{app}$) is not affected (Simnett et al., 1993b; Strauss et al., 1994). The transition into the force-generating state that is accelerated by EMD 53998 is associated with the release of P$_i$ from the actomyosin-ADP-P$_i$ ternary complex (Barth et al., 1995). Because EMD 53998 also antagonizes the inhibitory effect of inorganic phosphate in skinned fibers (Strauss et al., 1992a), it seems likely that EMD 53998 accelerates the P$_i$ release step, perhaps by lowering the P$_i$
affinity for myosin (Strauss et al., 1994). Therefore, its action should be antagonistic to that of BDM.

CGP 48506 (CGP), the (+)-enantiomer of the racemic mixture 1,5-benzodiazocine derivative 5-methyl-6-phenyl-1,3,5,6-tetrahydro-3,6-methano-1,5-benzodiazocine-2,4-dione, has been shown to shift the force-pCa curve to the left in porcine skinned right ventricular muscle fibers (Palmer et al., 1996). This action was paralleled by an increase in the ATPase activity. From these experiments it was concluded that CGP 48506 does not alter the apparent detachment rate of the cross-bridges (Palmer et al., 1996).

Ca²⁺-sensitizers may be advantageous for the treatment in human heart failure because they increase force of contraction without increasing the intracellular Ca²⁺ transient or energy expenditure. The kind of action by which EMD 57033 or CGP 48506 influence cross-bridge interaction in human failing myocardium is not fully understood. Thus, by studying the interference of EMD 57033 and CGP 48506 on the known inhibitory action of BDM on force generation in troponin I-depleted fibers from left ventricular human myocardium, the molecular mechanisms of EMD 57033 or CGP 48506 should be elucidated. To analyze the influence on cross-bridge kinetics, tension cost, i.e., the ratio of ATPase activity and tension development, was studied after application of EMD 57033, CGP 48506, and BDM.

Experimental Procedures

Myocardial Tissue. Experiments were performed on human left ventricular myocardium. Tissue was obtained during cardiac transplantation (n = 19, 8 female, 11 male; age 44 ± 4 years). Patients suffered from heart failure clinically classified as New York Heart Association class IV on the basis of clinical symptoms and signs as judged by the attending cardiologist shortly before operation. All patients gave written informed consent before surgery. Medical therapy consisted of diuretics, nitrates, angiotensin-converting enzyme inhibitors, and cardiac glycosides. Patients receiving catecholamines, β-adrenergic or Ca²⁺-antagonists were withdrawn from the study. Drugs used for general anesthesia were flunitrazepam and pancuronium bromide with isoflurane. Cardiac surgery was performed on cardiopulmonary bypass patients with cardiopulmonary arrest during hypothermia. The cardioplegic solution (a modified Bretschneider solution) contained 15 mM NaCl, 9 mM KCl, 4 mM MgCl₂, 180 mM histidine, 2 mM tryptophan, 30 mM mannitol, and 1 mM potassium dihydrogen oxoglutarate. The study was approved by the local ethics committee.

Skinned Fibers. Left ventricular muscle fibers were prepared according to previously published procedures (Schwinger et al., 1994). Briefly, the fiber bundles (diameter <0.2 mm) were dissected and permeabilized at 4°C for 20 h in a solution containing 50% (v/v) glycerol, 1% Triton X, and in 10 mM NaNO₃, 5 mM ATP, 5 mM MgCl₂, 4 mM EGTA, 2 mM 1,4-dithioerythritol, and 20 mM imidazole (pH 7.0). Afterward the fibers were stored in a similar solution but without Triton X at −20°C.

Troponin I Washout Experiments. The relaxation solution contained 20 mM imidazole, 10 mM ATP, 12.5 mM MgCl₂, 10 mM creatine phosphate, 5 mM NaNO₃, 5 mM EGTA, 1 mM 1,4-dithioerythritol, and 350 U/ml creatine kinase (pH 7.0). The ionic strength was adjusted to 130 mM by adding KCl. In the contraction solution EGTA was replaced by 5 mM calcium EGTA. The pCa (−log[Ca²⁺]) was varied by mixing contraction and relaxation solution as appropriate and pCa values were calculated according to Fabiato and Fabiato (1979), using the stability constants given by Fabiato (1981). For force measurements, fibers were mounted isometrically between a force transducer and a rigid post attached to a micrometer for length adjustment (Scientific Instruments, Heidelberg, Germany). In relaxation solution, fiber length was adjusted to an extent where resting tension was just threshold (slack position). An initial test contraction-relaxation cycle was performed on all fibers to ensure Ca²⁺ dependence of force in each preparation. After this procedure, fibers were again maximally contracted (pCa = 4.5) to establish a control level of maximum isometric tension. After a tension plateau had been reached, fibers were incubated for 10 min in relaxing solution containing 10 mM sodium vanadate. After incubation with vanadate, fibers were transferred to fresh relaxing solution to remove the vanadate. After washing out the vanadate, fibers were no longer Ca²⁺ regulated, i.e., they contracted maximally even at 10 nM free Ca²⁺. Experiments were performed according to Strauss et al. (1992a). Contracted fibers were then exposed to increasing concentrations of BDM (5–50 mM), and solutions containing BDM (5–50 mM) with and without the addition of EMD 57033 (10 μM), respectively, CGP 48506 (10 μM).

Immunocytochemistry in Skinned Fibers of Human Cardiac Muscle and Measurement of Sarcomere Length. Skinned fibers of failing hearts, prepared as described above, were used for immunocytochemical labeling of Z-lines by α-actinin staining. After three washes in 0.1 M PBS buffer, the skinned fiber preparations were incubated in a 1:800 dilution of mouse anti-rat α-actinin antibody for 1 h at room temperature, followed by treatment with a secondary biotinylated goat anti-mouse antibody (1:400) for 1 h and subsequent Cy3-labeled extravidin (1:600) for 1 h (Ji et al., 1999). Then the skinned fibers were washed with 0.1 M Tris-buffered saline and stored at −20°C until the sarcomere length measurement.

The measurement of sarcomeric length was performed using a Zeiss Axiovert 135 fluorescence microscope (filter set 15 Zeiss; excitation BP 546/16, emission LP 590), a Sony three chip camera, and computer-assisted imaging software (Optimas 6.01). For investigation of the sarcomeric length, the skinned fibers were fixed at slack position in relaxation solution. The distance of 10 to 15 actinin/Cy3-labeled Z-lines was measured at 10 different areas of each skinned fiber using a 40× Neofluor objective (Zeiss, Oberkochen, Germany). The sarcomeric length was calculated by dividing the measured distance by the number of spaces between labeled Z-lines. The mean of sarcomeric length for each skinned fiber was calculated from all investigated areas. Average sarcomere length was 2.01 ± 0.08 μm.

Force and ATPase Activity Measurements. In a second type of experiment, force and ATPase activity were simultaneously measured (Güth and Wojciechowski, 1986; experimental setup, Scientific Instruments). Relaxation solution contained 20 mM imidazole, 10 mM ATP, 5 mM NaNO₃, 5 mM EGTA, 12.5 mM MgCl₂, and 0.2 mM P₁,P₂-di(adenosine 5’) pentaphosphate. The contraction solution contained calcium EGTA (5 mM) instead of EGTA. The ATP concentration was stabilized with an ATP-regenerating system, phosphoenolpyruvate (12.5 mM), and pyruvate kinase (100 U/ml). ATPase activity and force were simultaneously measured using a linked NADH fluorescence assay (0.6 mM NADH, 140 U/lactate dehydrogenase). The relaxation solution contained 20 mM imidazole, 10 mM Na₅ATP, 5 mM NaNO₃, 5 mM EGTA, 12.5 mM MgCl₂, 5 mM phosphoenolpyruvate, 0.6 mM NADH, and 0.2 mM P₁,P₂-di(adenosine 5’) pentaphosphate (myokinase inhibitor), 25 mM cyclopiazonic acid, together with 100 units/ml pyruvate kinase and 125 units/ml lactate dehydrogenase. The contraction solution contained calcium EGTA (5 mM) instead of EGTA. Both solutions were mixed by a gradient mixer so that Ca²⁺ was successively increased every 15 s. Free Ca²⁺ concentration was determined by calculator programs designed for experiments in skinned muscle cells by Fabiato and Fabiato (1979). Measurement of developed tension and myosin ATPase activity started 3 s after the solution was exchanged. Developed tension and myosin ATPase activity had reached a stable plateau at that time. Experiments were performed as described previously (Brixius and Schwinger, 2000). All experiments were performed in slack position. By subtracting the basal ATPase activity obtained in the relaxation solution from the measured ATPase activity, the suprabasal ATP-
splitting rate was obtained. The ratio of suprabasal ATPase activity and force was assumed as a measure for the "tension cost".

**Materials.** EMD 57033 was generously provided by Merck (Darmstadt, Germany). CGP 48506 was a gift from Ciba-Geigy (Wehr, Germany). All other chemicals were of analytical grade or the best grade commercially available. Mouse anti-rat α-actinin antibody and Cy3-labeled extravidin were obtained from Sigma (Deisenhofen, Germany), and biotinylated goat anti-mouse antibody was from Dako Corp. (Carpinkia, Canada).

**Statistics.** All values are means ± S.E.M. unless otherwise noted. Student’s t test or paired t test were used to test significance. P values of <.05 were accepted as significant. pCa-force as well as pCa-myosin ATPase activity relationships were fitted by a modified Hill equation (Hill, 1910) as follows: \( Y = \frac{[Ca^{2+}]^n}{([Ca^{2+}] + IC_{50})^n} \), where \( Y \) is the fractional force, or myosin-ATPase activity, \( [Ca^{2+}] \) is the Ca\(^{2+}\) concentration giving half-maximal activation (inhibition), and \( H \) is an index of cooperativity (Hill coefficient). The concentration needed for half-maximal Ca\(^{2+}\) activation of tension development or myosin ATPase activity (EC\(_{50}\) for Ca\(^{2+}\)), the concentration of BDM needed to achieve a half-maximal decline of tension development (IC\(_{50}\) for BDM), all Hill-coefficients, and the tension cost (ratio of ATPase activity and tension development) were analyzed by GraphPad Prism (GraphPad, San Diego, CA).

**Results**

**BDM, EMD 57033, CGP 48506, and Tension Cost in Human Myocardium.** To investigate whether EMD or CGP may interact with the BDM-mediated influence on the cross-bridge detachment rate in human myocardium, Ca\(^{2+}\)-activated tension development and myosin-ATPase activity were simultaneously studied in the presence of BDM (10 mM), EMD (10 μM), and CGP (10 μM). All fibers were set in "slack position", i.e., fiber length was adjusted to an extent where resting tension was just threshold. In this situation a basal ATPase activity was measured of 78.7 ± 1.8 μmol ADP/s. Application of BDM (10 μM) increased Ca\(^{2+}\)-activated myosin ATPase activity by 64.5 ± 2.7 μmol ADP/s, whereas CGP (10 μM) did not change the ATPase activity (64 ± 7 μmol ADP/s) significantly decreased ATP\(_{max}\) (effect EMD, 84 ± 10 μmol ADP/s, N.S.). In skinned fiber preparations of human failing myocardium the EC\(_{50}\) Ca\(^{2+}\) of myosin ATPase activity was 1.22 μM (CI = 0.76–1.67 μM). EMD (10 mM) increased the Ca\(^{2+}\) concentration needed to achieve half-maximal myosin-ATPase activity (EC\(_{50}\) Ca\(^{2+}\) = 3.39 μM, CI = 2.77–4.02 μM, P < .05), as well as the slope of the ATPase-Ca\(^{2+}\)-curve (H\(_{ATP}\); control: 1.7 ± 0.15 + BDM (10 mM) = 2.7 ± 0.2). EMD 57033 (10 mM) did not change the Ca\(^{2+}\) sensitivity of the myosin-ATPase activity (EC\(_{50}\) Ca\(^{2+}\) = 1.39 μM, CI = 0.3–2.27 μM) and H\(_{ATP}\) (2.1 ± 0.4 μmol ADP/s), whereas CGP 48506 (10 μM) induced a leftward shift of myosin ATPase activity (EC\(_{50}\) Ca\(^{2+}\) = 0.62 μM, CI = 0.53–0.72 μM) and a significant increase of H\(_{ATP}\) (3.0 ± 0.4). When comparing the leftward shift of contractile Ca\(^{2+}\) sensitivity (~1.04 μM), CGP induced a smaller leftward shift of the Ca\(^{2+}\) sensitivity of the myosin-ATPase activity (~0.59 μM).

Figure 2 presents the influence of BDM, EMD 57033, and CGP 48506 on the Ca\(^{2+}\)-dependent changes of maximal Ca\(^{2+}\)-activated myosin ATPase activity (Fig. 2A) and the Ca\(^{2+}\)-ATPase curves (Fig. 2B). Under basal conditions, maximal Ca\(^{2+}\)-activated myosin ATPase activity (ATP\(_{max}\)) was 102 ± 16 μmol ADP/s. BDM (10 mM, 52 ± 5 μmol ADP/s) and CGP (10 μM, 64 ± 7 μmol ADP/s) significantly decreased ATP\(_{max}\) (effect EMD, 84 ± 10 μmol ADP/s, N.S.). In skinned fiber preparations of human failing myocardium the EC\(_{50}\) Ca\(^{2+}\) of myosin ATPase activity was 1.22 μM (CI = 0.76–1.67 μM). EMD (10 mM) increased the Ca\(^{2+}\) concentration needed to achieve half-maximal myosin-ATPase activity (EC\(_{50}\) Ca\(^{2+}\) = 3.39 μM, CI = 2.77–4.02 μM, P < .05), as well as the slope of the ATPase-Ca\(^{2+}\)-curve (H\(_{ATP}\); control: 1.7 ± 0.15 + BDM (10 mM) = 2.7 ± 0.2). EMD 57033 (10 mM) did not change the Ca\(^{2+}\) sensitivity of the myosin-ATPase activity (EC\(_{50}\) Ca\(^{2+}\) = 1.39 μM, CI = 0.3–2.27 μM) and H\(_{ATP}\) (2.1 ± 0.4 μmol ADP/s), whereas CGP 48506 (10 μM) induced a leftward shift of myosin ATPase activity (EC\(_{50}\) Ca\(^{2+}\) = 0.62 μM, CI = 0.53–0.72 μM) and a significant increase of H\(_{ATP}\) (3.0 ± 0.4). When comparing the leftward shift of contractile Ca\(^{2+}\) sensitivity (~1.04 μM), CGP induced a smaller leftward shift of the Ca\(^{2+}\) sensitivity of the myosin-ATPase activity (~0.59 μM).

Figure 3 summarizes the results obtained for tension cost. Application of BDM (10 mM) resulted in an increase of tension cost compared with control, indicating that BDM increases the cross-bridge detachment rate. The opposite holds true for the Ca\(^{2+}\) sensitizers EMD and CGP; the tension cost was decreased after application of both the Ca\(^{2+}\) sensitizers.
(10 μM), which means that they may decrease the cross-bridge detachment rate.

**Force Development of Troponin I-Depleted Fibers.**

Figure 4 presents an original tracing of the force development before and after troponin I depletion by vanadate extraction in skinned fiber preparations of human myocardium. Initially, all fibers demonstrated a Ca\(^{2+}\)-dependent contraction. After incubation in a solution of 10 mM vanadate and transfer to normal relaxation solution without vanadate, a force development was observed, which was 93% of the \(F_{\text{max}}\) measured before the vanadate treatment. Under the same conditions, myosin ATPase activity was 93% of the \(ATP_{\text{max}}\) measured before vanadate treatment. No additional Ca\(^{2+}\)-dependent force or myosin ATPase activity was obtained when the free Ca\(^{2+}\) concentration was increased in the solution. Thus, Ca\(^{2+}\)-dependent cross-bridge-interaction was no longer present in these fibers.

**Effect of BDM, EMD 57033, and CGP 48506 on Ca\(^{2+}\)-Independent Tension in Troponin I-Depleted Fibers.**

To investigate whether the negative inotropic effect of BDM can be directly attributed to an interaction of BDM with the cross-bridge cycle in human myocardium, the concentration-dependent effect of BDM (5–50 mM) was studied in troponin I-depleted, Ca\(^{2+}\)-unregulated skinned fibers. The results are summarized in Fig. 5. Contractile force of the vanadate-treated, troponin I-depleted cardiac fibers was concentration dependently decreased by BDM. The concentration of BDM needed to achieve a 50% decrease in basal force (IC\(_{50}\)) was 7.22 mM (CI = 4.41–10.0 mM). The negative inotropic action of BDM was partly antagonized by the application of EMD 57033 (10 μM); EMD 57033 produced a significant rightward shift of the BDM concentration-response curve [IC\(_{50}\) of BDM in the presence of EMD 57033 (10 μM) = 19.97 mM, CI = 17.59–22.36 mM, \(P < .05\)]. EMD did not change the slope of the force-BDM curve. In the absence of BDM, EMD 57033 increased maximal developed tension in troponin I-depleted fibers by +22.8 ± 4.0% (\(P < .05\)). In the presence of CGP 48506 (10 μM) a significant rightward shift of the BDM concentration-response curve was observed [IC\(_{50}\) of BDM in the presence of CGP 48506 (10 μM) = 15.30 mM, CI = 12.83–17.77 mM, Fig. 5]. CGP 48506 did not influence the slope of the BDM-force curve. CGP decreased \(F_{\text{max}}\) in troponin I-depleted fibers by −11.1 ± 1.8%, in the absence of BDM.

**Discussion**

Ca\(^{2+}\) sensitizers may be advantageous for the treatment in human heart failure because they increase force of contraction without increasing the intracellular Ca\(^{2+}\) transient or energy expenditure. The mode of action of the novel developed Ca\(^{2+}\) sensitizers EMD and CGP in human failing myocardium is yet not fully understood. In contrast to these Ca\(^{2+}\) sensitizers, the nucleophilic oxime BDM has been shown to depress contractile force independently of changes in intracellular calcium (Backx et al., 1994). From simultaneous measurements of force and myosin ATPase activity in skinned cardiac rat trabeculae, it has been concluded that BDM causes a marked increase in the apparent rate of cross-bridge detachment (Ebus and Stienen, 1996). Therefore, to further understand the mechanism of action of Ca\(^{2+}\) sensitizers, the present study investigated whether the inhibitory
action of BDM could be antagonized by the addition of the Ca\textsuperscript{2+} sensitzers EMD or CGP to human myocardium. For the direct study of cross-bridge-interaction troponin I-depleted skinned fiber preparations were used, in which a Ca\textsuperscript{2+}-unregulated tension development is taking place (Strauss et al., 1992b). To analyze the influence of EMD, an effect of BDM on cross-bridge kinetics, tension cost (ratio of maximum tension development by EMD is paralleled by an unchanged maximum Ca\textsuperscript{2+}-activated myosin ATPase activity compared with control conditions (in the absence of EMD, Table 1). These results may indicate that EMD increases the probability of the cross-bridges to be in the force-generating state, thereby increasing the force per cross-bridge. Accordingly, there are no observable changes in cross-bridge kinetics or fiber stiffness of rabbit fast skeletal myofilaments (Kraft et al., 1994). In chemically skinned cells from rat ventricle EMD induced marked increases in the rate of tension redevelopment (\(k_{\text{tr}}\)) after brief slack release/restretch (Vannier et al., 1997). This indicates the existence of an increased number of attached cross-bridges during one cycle. An effect of EMD on the distribution of force-producing, preforce- and nonforce-producing cross-bridges has been also proposed (Strauss et al., 1994).

In a previous study, it was suggested that EMD binds to troponin C and may modulate its Ca\textsuperscript{2+}-dependent interaction with troponin I, and consequently, alter the cooperative properties of the thin filaments (Pan and Johnson, 1996). In the present study, application of EMD did not change the Hill coefficient of the Ca\textsuperscript{2+}-force or the Ca\textsuperscript{2+}-ATPase relationship, indicating that EMD does not alter the Ca\textsuperscript{2+} affinity of troponin C. Consistently, the Ca\textsuperscript{2+}-sensitizing effect of EMD was also present in troponin I-depleted, Ca\textsuperscript{2+}-unregulated skinned fiber preparations, indicating that the Ca\textsuperscript{2+}-sensitizing effect of EMD is, at least partially, independent from an interaction with troponin C/troponin I-mediated mechanisms. This is in accordance with studies in Triton X-skinned fibers from canine heart, in which EMD did not influence Ca\textsuperscript{2+} binding to troponin C (Solaro et al., 1993).

**Effect of EMD 57033 on Cross-Bridge Cycling.** EMD decreases tension cost in human failing myocardium and additionally increases maximum tension development (Table 1). Additionally, as shown in the present study, the increase of maximum tension development by EMD is paralleled by an unchanged maximum Ca\textsuperscript{2+}-activated myosin ATPase activity (Fig. 5). In chemically skinned cells from rat ventricle, application of EMD induced marked increases in the rate of tension redevelopment (\(k_{\text{tr}}\)) after brief slack release/restretch (Vannier et al., 1997). This indicates the existence of an increased number of attached cross-bridges during one cycle. An effect of EMD on the distribution of force-producing, preforce-, and nonforce-producing cross-bridges has been also proposed (Strauss et al., 1994).

**Effect of CGP 48506 on Cross-Bridge Cycling.** In the present study obtained in left ventricular Triton X-skinned fiber preparations of human failing myocardium, CGP significantly decreased maximal Ca\textsuperscript{2+}-activated myosin ATPase activity without depressing maximal Ca\textsuperscript{2+}-activated force (Table 1), which may indicate that the Ca\textsuperscript{2+}-sensitizing effect of CGP is partly due to an increase of the force development per cross-bridge. In contrast to EMD, the Ca\textsuperscript{2+}-sensitizing effect of CGP is accompanied by an increased Ca\textsuperscript{2+} sensitiv-

**TABLE 1**

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**Fig. 4.** Original tracing of the isometric force development during the troponin I washout experiments. After application of BDM in skinned fiber preparations of human failing myocardium. After testing the Ca\textsuperscript{2+} dependence with an initial maximal contraction (free Ca\textsuperscript{2+} 11.5 \(\mu\)M), followed by a subsequent relaxation (free Ca\textsuperscript{2+} 10 \(nM\)), the skinned fibers were incubated in vanadate (10 mM) to extract troponin I. After vanadate (10 mM) treatment, Ca\textsuperscript{2+}-dependent contraction was lost indicated by a maximal contraction of the fiber even at very low Ca\textsuperscript{2+} concentrations (10 mM). Contracted fibers were then immersed into solutions with increasing concentrations of BDM (5–50 mM), resulting in a decrease of the developed tension.

**Fig. 5.** The inhibitory effect of BDM (□) on troponin I-depleted skinned cardiac fibers of human failing myocardium in the presence of EMD 57033 (10 \(\mu M\), ▲, \(n = 9\)) or CGP 48506 (10 \(\mu M\), ◄, \(n = 9\)).
ity of the myosin ATPase. There are two hypotheses for the Ca\textsuperscript{2+}-sensitizing mechanism of CGP proposed (Palmer et al., 1996). First, the Ca\textsuperscript{2+}-sensitizing mechanism of CGP may promote the change from the detached to the weakly attached cross-bridge state, thereby shifting a greater proportion of the cross-bridges ready to shift to the force-producing state when actomyosin interaction is promoted through Ca\textsuperscript{2+} binding to troponin C. This hypothesis may supported by the finding of the present study that CGP 48506 changes the Ca\textsuperscript{2+} sensitivity of both force and myosin ATPase activity. Consistently, we found an increased Hill coefficient for both the Ca\textsuperscript{2+} force and the ATPase-Ca\textsuperscript{2+} relationship, indicating that CGP increases the cooperativity of the myofilaments and thereby improves the Ca\textsuperscript{2+} affinity of troponin C. However, as shown by the present study, the Ca\textsuperscript{2+}-sensitizing effect of CGP was also present in Ca\textsuperscript{2+} and thus troponin C)-unregulated cardiac muscle fibers. Therefore, additional mechanisms seem to contribute to the Ca\textsuperscript{2+}-sensitizing effect of CGP. CGP significantly reduced tension cost in human failing myocardium. Because alterations of tension cost reflect changes of the cross-bridge detachment rate, the present study supports the hypothesis of Herold et al. (1995) that CGP 48506 increases Ca\textsuperscript{2+} sensitivity by a reduction of the dissociation constant g\textsubscript{app}.

**Antagonistic Effects of EMD 57033 and CGP 48506 on BDM-Mediated Depression of Cross-Bridge Cycling.** The inhibitory effect of BDM in troponin I-depleted skinned fiber preparations from human myocardium was antagonized by the Ca\textsuperscript{2+} sensitizers EMD and CGP. These results suggest that the action of EMD and CGP, like that of BDM, occur at the level of the actin cross-bridge reaction (Wolska et al., 1996). Consistently, it has been shown that EMD 53998 accelerates the transition into the force-generating state by increasing the release of P\textsubscript{i} from the actomyosin-ADP-P\textsubscript{i} ternary complex. This mechanism may also hold true for EMD 57033. EMD 57033 may enhance the stability of the attached cross-bridge state, thereby slowing the rate of detachment and increasing the rate of attachment. These actions may reverse the suppressive actions of BDM on the cross-bridge cycle, which have been attributed to a depression in the number of force-generating cross-bridges as well as to a facilitation of P\textsubscript{i} release in the conversion of the weakly attached state into the force-generating state (Gwathmey et al., 1991).

The antagonistic action of CGP on BDM-mediated force suppression may be explained by the findings showing that BDM slows the rate of P\textsubscript{i} release (Zhao et al., 1995), and thus the transition of the nonforce-generating cross-bridge into force-generating ones. Consistently, CGP may effect a cross-bridge state subsequent to the P\textsubscript{i} release but preceding the state immediately before detachment, for example, by inhibition of the release of ADP from the actomyosin-ADP complex, thereby prolonging the force-generating state of the cross-bridges (Palmer et al., 1996).

However, from the present studies, it cannot be concluded whether EMD and CGP exert their effects on distinct steps of the cross-bridge cycle from BDM. They could also simply enhance the stability of the attached cross-bridge state, thereby slowing the rate of detachment and increasing the rate of attachment. This would mean that EMD and CGP may just reverse the effects of BDM. The effects of CGP and EMD can be tested in further experiments by varying the levels of various substrates and products (i.e., ATP, ADP, and P\textsubscript{i}). In conclusion, the present study indicates that the Ca\textsuperscript{2+}-sensitizing effect of EMD and CGP results from interaction at different stages of the cross-bridge cycle. Both Ca\textsuperscript{2+} sensitizers decrease the energy consumption for force in human failing myocardium but only EMD 57033 increases maximal force development and only CGP was effective to reduce maximal ATPase consumption.

**Limitations of the Present Study.** It cannot be excluded that diseased human myocardium taken from explanted hearts may show intramyocardial damage. However, special care was taken to check the used tissue by electron microscopic techniques. Figure 6 shows an electronic microscopic picture taken from a Triton X-skinned fiber preparation of human left ventricular myocardium.
the presented experimental studies. This picture shows distinct sarcomeric structures, so that detrimental experimental damage to the preparation can be excluded. In addition, all studies of this publication are done in failing human myocardium. This article does not intend to work out differences in the action of EMD 57033 and CGP 48506 between normal and diseased states because pharmacological treatment is focused on failing hearts.

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