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In vivo evidence that EMD 57033 restores myocardial responsiveness to intracoronary Ca^{2+} in stunned myocardium

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Abstract

Despite ample in vitro evidence that myofilament Ca^{2+}-responsiveness of stunned myocardium is decreased, in vivo data are inconclusive. Conversely, while Ca^{2+}-sensitizing agents increase myofilament Ca^{2+}-responsiveness in vitro, it has been questioned whether this also occurs in vivo. We therefore tested in open-chest anesthetized pigs whether EMD 57033 (the (+) enantiomer of 5-[1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one) increases responsiveness to Ca^{2+} of non-stunned myocardium and restores function of stunned myocardium by normalizing the responsiveness to Ca^{2+}. Studies were performed under β-adrenoceptor blockade to minimize the contribution of the phosphodiesterase-III inhibitory actions of EMD 57033. Consecutive intracoronary Ca^{2+} infusions were used to evaluate the contractile response (assessed by the left ventricular end-systolic elastance, E_{es}) to added Ca^{2+} of non-stunned myocardium and myocardium stunned by 15 min coronary artery occlusion and 30 min reperfusion. In non-stunned propranolol-treated myocardium, the Ca^{2+} infusions doubled E_{es} (baseline 6.9 ± 0.9 mmHg mm^{-2}, $n = 8$). Following Ca^{2+}-washout, subsequent EMD 57033 infusion (0.1 mg kg^{-1} min^{-1}, i.v.) tripled E_{es} ($P < 0.05$) and potentiated the Ca^{2+}-induced increase in E_{es} to 55.7 ± 10.0 mmHg mm^{-2} ($P < 0.05$). Stunning ($n = 7$) decreased E_{es} to 5.3 ± 0.6 mmHg mm^{-2} ($P > 0.10$) and attenuated the Ca^{2+}-induced increase in E_{es} ($P < 0.05$). Subsequent infusion of EMD 57033 increased E_{es} to 6.8 ± 1.8 mmHg mm^{-2} ($P < 0.05$) and restored responsiveness to added Ca^{2+}. These in vivo findings are consistent with the in vitro observations that myofilament Ca^{2+}-responsiveness of stunned myocardium is reduced and that EMD 57033 increases contractility by enhancing myofilament Ca^{2+}-responsiveness.

Keywords: Ca^{2+}-responsiveness; Ca^{2+}-sensitizer; Myocardial contractility; End-systolic elastance; Stunning

1. Introduction

It has been shown in in vitro models that a reduced responsiveness of the myofilaments to Ca^{2+} underlies the mechanism of myocardial stunning (Gao et al., 1995), but evidence that this mechanism is also operative in vivo is lacking (see Duncker et al., 1998; Bolli and Marban, 1999). For instance, Ito et al. (1987) observed that in an in vivo canine model of regional myocardial stunning, maximum systolic shortening attainable with intracoronary Ca^{2+} infusions was not different for stunned and normal myocardium. In addition, Heusch et al. (1996) also failed to find evidence for a decreased Ca^{2+} responsiveness, as assessed by the response of a regional work index to intracoronary Ca^{2+} in in vivo porcine myocardium, stunned by a 90-min flow reduction that decreased the local myocardial work index by 60%, a protocol that produced stunning without necrosis. A complicating factor in explaining the discrepancies between the in vitro and the in vivo results, is that in the in vivo studies, contractile function was estimated using indices that display considerable load-dependency, and of which it is known that their load-dependency increases with stunning (Fan et al., 1995). Thus, Hofmann et al. (1993) showed under well controlled in vitro conditions that pCa for half maximal activation of

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tension was decreased, compared to baseline, in single cell-sized preparations from porcine myocardium stunned in vivo by a 45-min period of 60% coronary flow reduction and 30 min of reperfusion. We have shown earlier that EMD 60263, a thiadiazinone derivative, which has been demonstrated to possess Ca^{2+}-sensitizing properties in vitro (Ravens et al., 1996; Bezstarosti et al., 1997), is capable of restoring systolic shortening of regionally stunned myocardium in vivo (Soei et al., 1994). The beneficial effects of a number of Ca^{2+}-sensitizing agents on systolic function of stunned myocardium have now been confirmed in several isolated and intact heart studies (Korbmacher et al., 1994, 1997; Abe et al., 1995; De Zeeuw et al., 2000), but in none of these studies was it actually shown that during stunning, the responsiveness to Ca^{2+} was altered and that subsequent administration of the Ca^{2+}-sensitizing agents restored the responsiveness to Ca^{2+}.

Therefore, the aims of the present in vivo study were to determine whether administration of a Ca^{2+}-sensitizing agent alters the myocardial responsiveness to added Ca^{2+} in non-stunned myocardium and whether this agent restores function of stunned myocardium by normalization of the myocardial responsiveness to Ca^{2+}. As a Ca^{2+}-sensitizing agent, we used EMD 57033 (the (+) enantiomer of 5-[1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one), because we have shown that this agent, in a dose of 0.2 mg kg^{-1} min^{-1}, is capable of restoring systolic function of regionally stunned myocardium, without adversely affecting diastolic function (De Zeeuw et al., 2000), a potential concern with this class of agents (Hajjar and Gwathmey, 1991; Soei et al., 1999). Because EMD 57033 possesses minor phosphodiesterase-III (PDE-III) inhibitory properties (White et al., 1993; Ravens et al., 1996), the effect of EMD 57033 was evaluated in the presence of the β-adrenoceptor antagonist propranolol to minimize tonic and stimulated cAMP production, thereby keeping the contribution of PDE-III inhibition to the actions of EMD 57033 to a minimum. The myocardial responsiveness to added Ca^{2+} was determined using intracoronary infusions to prevent that changes in systemic hemodynamics act as confounding factors. Finally, to further minimize the influence of changes in loading conditions, we used, in analogy to the left ventricular end-systolic pressure (LVESP)–volume (Suga, 1990) and LVESP–segment length (Aversano et al., 1986; Fan et al., 1995) relations, the LVESP–segment area relation to evaluate the contractile response to Ca^{2+}.

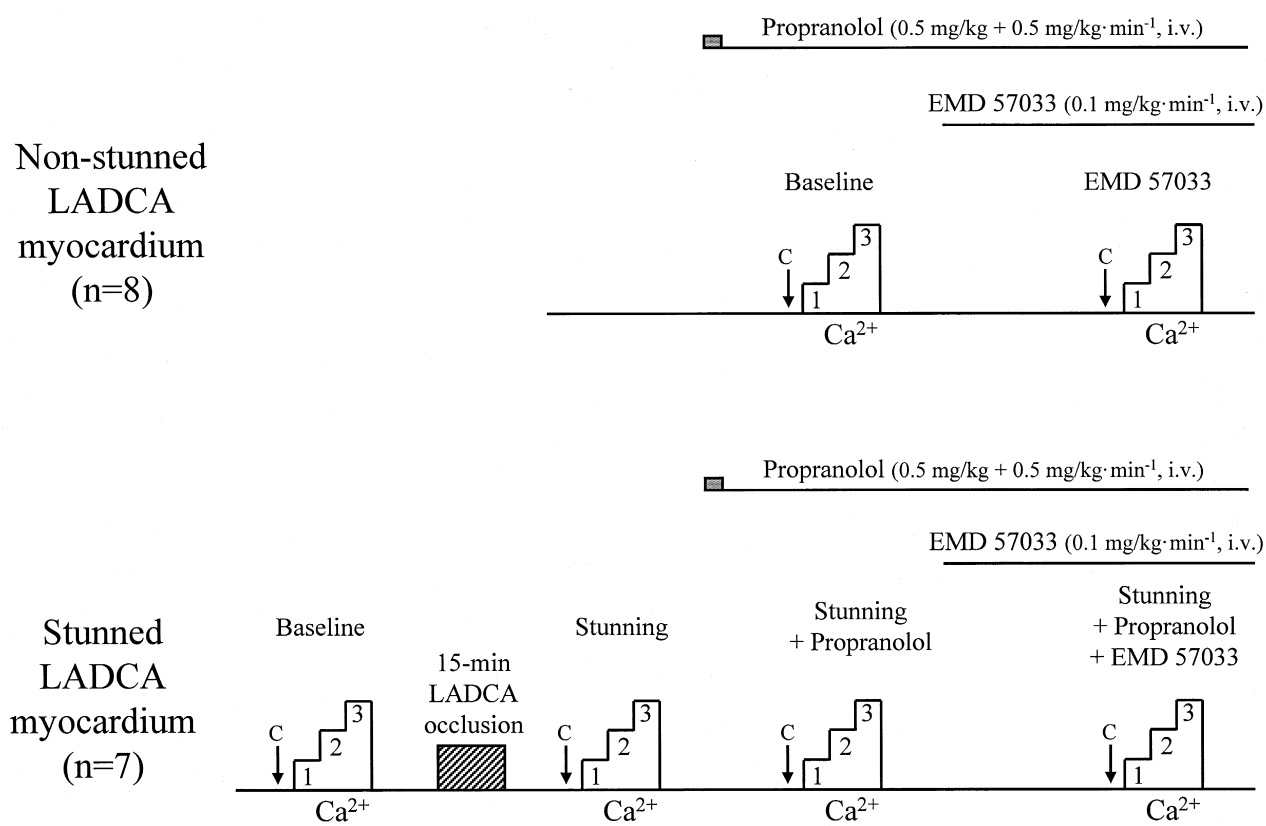

Fig. 1. Experimental protocols for propranolol-treated pigs with non-stunned myocardium and pigs with regionally stunned myocardium. Stunning was produced by a 15-min LADCA occlusion and 30 min of reperfusion. Three consecutive Ca^{2+} infusions into the LADCA were administered for 5 min each at rates of 18, 36 and 54 μmol min^{-1}, respectively. The arrows indicate the control (C) measurements before the start of the Ca^{2+} infusions (Tables 1 and 2).

2. Methods

Experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" of the Council of the American Physiological Society and under the regulations of the Erasmus University Rotterdam.

2.1. Animal preparation

Fifteen with ketamine (20–30 mg kg^{-1}, i.m.) sedated cross-bred Landrace–Yorkshire pigs (25–35 kg) were anesthetized with sodium pentobarbital (20 mg kg^{-1}, i.v.), intubated and ventilated with oxygen-enriched air (Soei et al., 1999; De Zeeuw et al., 2000). Fluid-filled catheters were inserted for intravenous administration of sodium pentobarbital (5–10 mg kg^{-1} h^{-1}) and fluids, and for measurement of arterial blood pressure. A micromanometer-tipped catheter (B. Braun Medical) was inserted for monitoring left ventricular blood pressure. A balloon catheter was positioned in the inferior caval vein to transiently decrease left ventricular preload. After administration of pancuronium bromide (4 mg, i.v.), a midsternal thoracotomy was performed and an electromagnetic flow probe (Skalar) was placed around the ascending aorta, while a Doppler flow probe (Triton Technology) was placed proximally on the left anterior descending coronary artery (LADCA). Distal to the flow probe, the LADCA was dissected free for placement of an atraumatic clamp and cannulated for local infusion of Ca^{2+}. Segment area was measured using sonomicrometry (Triton Technology) by placing ultrasonic crystals in the midmyocardial layer approximately 10 mm apart in the distribution areas of the LADCA and the left circumflex coronary artery (LCXCA). To minimize the influence of malalignment of a single crystal pair with the fiber direction, two pairs of crystals were implanted in each region: one pair parallel and another pair perpendicular to the myocardial fiber direction.

2.2. Experimental protocols

2.2.1. Propranolol-treated pigs with non-stunned myocardium

To evaluate the effect of EMD 57033 on the responsiveness to intracoronary Ca^{2+} of non-stunned myocardium, eight pigs received propranolol (0.5 mg kg^{-1} + 0.5 mg kg^{-1} h^{-1}, a dose that inhibits the isoproterenol-induced increases in heart rate and left ventricular dP/dt_{max} by more than 90% in pigs, Wolffenbuttel and Verdouw, 1983), after which hemodynamic variables were recorded and LVESP–segment area relations were constructed by transiently reducing preload (De Zeeuw et al., 2000). Then, three consecutive 5-min infusions of Ca^{2+} ($CaCl_2 \cdot 2H_2O$ dissolved in saline) were administered into the LADCA at rates of 0.25, 0.50 and 0.75 ml min^{-1} (corresponding to 18, 36 and 54 μmol min^{-1}, respectively). At the end of each infusion step, measurements were repeated (Fig. 1). Following Ca^{2+}-washout, the EMD 57033 infusion (0.1 mg kg^{-1} min^{-1}, i.v.) was started and after 30 min, the Ca^{2+} infusions were repeated.

2.2.2. Pigs with stunned myocardium

To evaluate the effect of EMD 57033 on the myocardial responsiveness to intracoronary Ca^{2+} during stunning, seven pigs received the intracoronary Ca^{2+} infusions before (baseline) and 30 min after a 15-min LADCA occlusion (stunning; Fig. 1). Subsequently, the β-adrenoceptors were blocked by propranolol and after repeating the Ca^{2+} infusions (stunning + propranolol), the EMD 57033 infusion was started and 30 min later, the myocardial responsiveness to intracoronary Ca^{2+} was again determined (stunning + propranolol + EMD 57033).

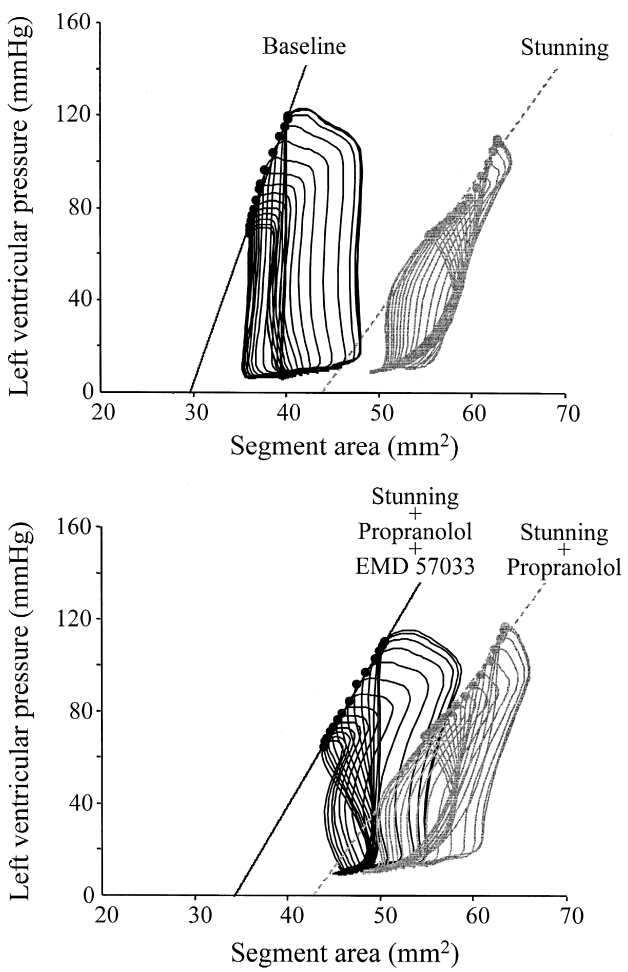

Fig. 2. Example of LVESP–segment area relations of the LADCA region during baseline, stunning, stunning + propranolol and stunning + propranolol + EMD 57033 in an individual pig, in which the myocardium was stunned by a 15-min occlusion of the LADCA.

2.3. Data analysis

All data were digitized and stored for off-line analysis (De Zeeuw et al., 2000). Segment length data were normalized to an end-diastolic length of 10 mm at baseline to correct for variability in the implantation distance between the various crystal pairs in the different animals. Regional left ventricular end-systolic elastance (E_{es}) was assessed using LVESP–segment area relations obtained from the relation between the left ventricular pressure and the segment area encompassed by the two pairs of crystals. The slope of the LVESP–segment area relations (end-systolic elastance, E_{es}) and the zero pressure–area intercept (A_0) were determined via linear regression analysis of the LVESP–segment area data points (Fig. 2), which were obtained using an iterative method (Van der Velde et al., 1991). Data are mean ± S.E.M. Statistical significance ($P < 0.05$, two-tailed) of changes was determined using one-way or two-way analysis of variance. Post-hoc testing was performed using Dunnett's test.

3. Results

3.1. Hemodynamics

3.1.1. Propranolol-treated pigs with non-stunned myocardium

In the propranolol-treated pigs, there were no changes in any of the hemodynamic parameters during the intracoronary Ca^{2+} infusions (not shown), except for a dose-dependent increase in left ventricular dP/dt_{max} up to 17 ± 4% ($P < 0.05$). The infusion of EMD 57033, which was started after recovery from the Ca^{2+} infusions, increased left ventricular dP/dt_{max} by 28 ± 8% and heart rate by 7 ± 3% (both $P < 0.05$; Table 1). The subsequent intracoronary Ca^{2+} infusions had again no hemodynamic effects (not shown), except for the increase in left ventricular dP/dt_{max} by 27 ± 5% ($P < 0.05$).

3.1.2. Pigs with stunned myocardium

When the β-adrenoceptors were still unblocked, the intracoronary Ca^{2+} infusions only increased left ventricular dP/dt_{max} (up to 17 ± 4%, $P < 0.05$). Production of myocardial stunning after recovery from the Ca^{2+} infusions, was accompanied by decreases in mean arterial blood pressure (9 ± 3%), cardiac output (13 ± 5%) and left ventricular dP/dt_{max} (18 ± 5%), while heart rate and systemic vascular resistance remained unchanged (Table 1). Similar to the non-stunned myocardium, the intracoronary Ca^{2+} infusions increased left ventricular dP/dt_{max} dose-dependently by up to 13 ± 5%, while the other parameters were not affected. In the presence of propranolol, infusion of Ca^{2+} into the stunned myocardium again affected only left ventricular dP/dt_{max} (24 ± 5%). The subsequent infusion of EMD 57033 increased heart rate (4 ± 2%), cardiac output (21 ± 8%) and left ventricular dP/dt_{max} (30 ± 10%), while left ventricular end-diastolic pressure decreased slightly (all $P < 0.05$). In the presence of EMD 57033, the only effects of the intracoronary Ca^{2+} infusions on hemodynamics were an increase in left ventricular dP/dt_{max} (27 ± 3%, $P < 0.05$) and a small decrease (12 ± 2%, $P < 0.05$) in mean arterial pressure.

Table 1
Effect of EMD 57033 on control values of global hemodynamics of propranolol-treated pigs with non-stunned myocardium and of pigs with stunned myocardium

	Propranolol-treated pigs with non-stunned myocardium ($n = 8$)		Pigs with stunned myocardium ($n = 7$)			
	Baseline	EMD 57033	Baseline	Stunning	Stunning + propranolol	Stunning + propranolol + EMD 57033
HR (bpm)	101 ± 7	107 ± 6[a]	106 ± 6	109 ± 5	91 ± 5[b]	94 ± 4[c]
MAP (mmHg)	86 ± 4	83 ± 6	94 ± 4	85 ± 4[d]	70 ± 5[b]	76 ± 4
CO (l min^{-1})	2.7 ± 0.2	2.9 ± 0.3	3.3 ± 0.3	2.8 ± 0.3[d]	2.6 ± 0.3	3.1 ± 0.3[c]
SVR (mmHg l^{-1} min^{-1})	33 ± 3	31 ± 3	30 ± 3	32 ± 3	28 ± 3[b]	25 ± 2
LV$dP/dt_{max}$ (mmHg s^{-1})	1360 ± 80	1720 ± 100[a]	1610 ± 80	1320 ± 70[d]	1010 ± 100[b]	1270 ± 90[c]
LVEDP (mmHg)	3.3 ± 0.8	2.1 ± 0.8	4.4 ± 1.3	6.9 ± 1.4[d]	7.3 ± 1.5	5.0 ± 1.5[c]
CBF (ml min^{-1})	28 ± 4	32 ± 3	22 ± 2	20 ± 3	20 ± 3	36 ± 6[c]

HR, heart rate; MAP, mean arterial blood pressure; CO, cardiac output; SVR, systemic vascular resistance; LV$dP/dt_{max}$, maximal rate of rise in left ventricular pressure; LVEDP, left ventricular end diastolic pressure; CBF, coronary blood flow. Values are mean ± S.E.M.

[a] $P < 0.05$ EMD 57033 vs. baseline.

[b] $P < 0.05$ stunning + propranolol vs. stunning.

[c] $P < 0.05$ stunning + propranolol + EMD 57033 vs. stunning + propranolol.

[d] $P < 0.05$ stunning vs. baseline.

3.2. Left ventricular end systolic pressure–segment area relations

3.2.1. Propranolol-treated pigs with non-stunned myocardium

Infusion of Ca^{2+} caused a dose-dependent counter clockwise rotation of the LVESP–segment area relation of the myocardium, perfused by the LADCA (Fig. 3A), thereby almost doubling E_{es} (Fig. 4A), without affecting A_o (27 ± 3 and 29 ± 2 mm^2 during control and during the highest Ca^{2+} infusion rate, respectively). After recovery from the Ca^{2+} infusions, the subsequent infusion of EMD 57033 more than doubled E_{es} (Table 2) and enhanced the Ca^{2+}-induced increments in E_{es} (Figs. 3A and 4A) without affecting A_o (30 ± 1 and 31 ± 1 mm^2 during control and during the highest Ca^{2+} infusion rate, respectively).

The intravenous administration of EMD 57033 also caused a doubling of E_{es} in the myocardium perfused by the LCXCA (Table 2). However, in this segment, the Ca^{2+} infusions into the LADCA before or during the EMD 57033 infusions altered neither E_{es} ($P = 0.28$ and $P = 0.09$; Fig. 4B) nor A_o ($P = 0.47$ and $P = 0.79$, not shown). These results indicate that the Ca^{2+} infusions into the LADCA did not lead to a spillover of Ca^{2+} in the distribution area of the LCXCA and that the ongoing infusion of EMD 57033 itself did not further increase contractility during the Ca^{2+} infusions.

3.2.2. Pigs with stunned myocardium

Infusion of Ca^{2+} during baseline also caused a counter clockwise rotation of the LVESP–segment area relation (Fig. 3B), thereby more than doubling E_{es} of the myocardium perfused by the LADCA (Fig. 4C) without affecting A_0 (not shown). Myocardial stunning lowered E_{es} from 6.6 ± 1.2 to 5.3 ± 0.6 mmHg mm^{-2} ($P > 0.10$), and caused a rightward shift of the LVESP–segment area relation reflected by the increase in A_0 from 24 ± 4 to 44 ± 2 mm^2 ($P < 0.05$; Table 2). The Ca^{2+} infusions in the stunned myocardium caused a leftward shift of the

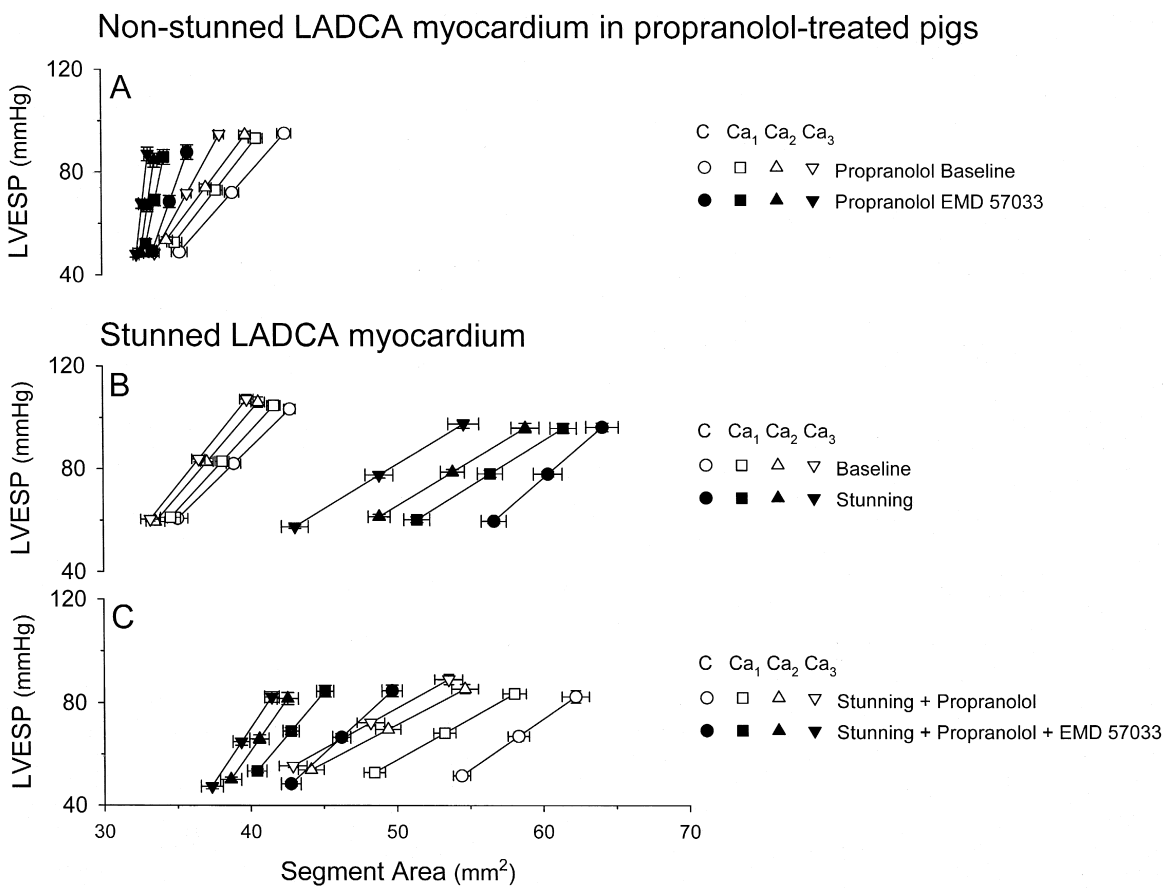

Fig. 3. Effect of EMD 57033 (0.1 mg kg^{-1} min^{-1} i.v.) on the response to three Ca^{2+} infusions into the LADCA of the LVESP–segment area relation in the distribution area of the LADCA of non-stunned (panel A) and stunned (panels B and C) in vivo porcine myocardium. The three data points in each curve were derived from the linear LVESP–segment area relation by computing LVESP at three segment areas within the range of actual measurements (minimum, maximum and [minimum + maximum]/2) for each animal. C = control; Ca_1, Ca_2 and Ca_3 refer to the three Ca^{2+} infusion rates of 18, 36 and 54 μmol min^{-1}, respectively.

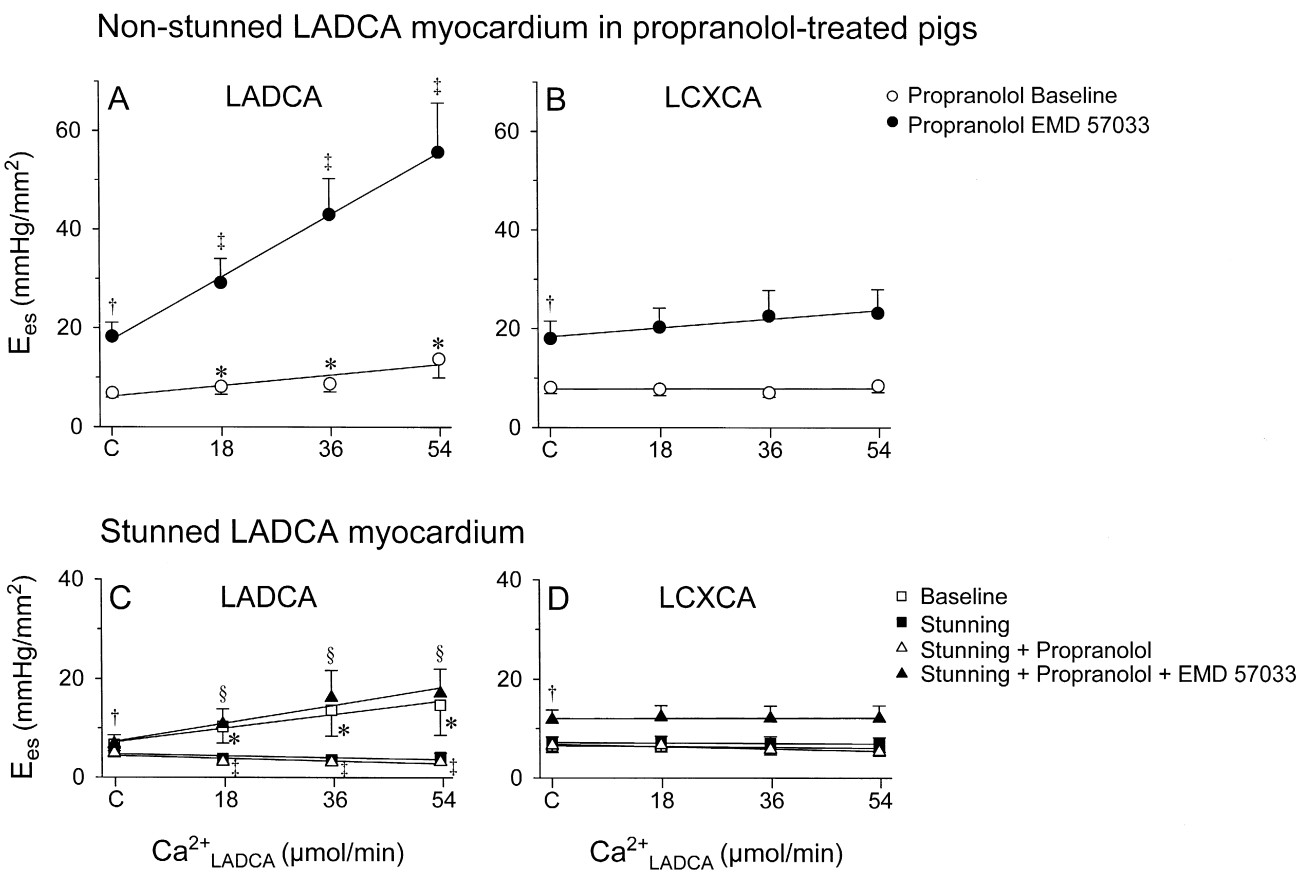

Fig. 4. Effect of EMD 57033 (0.1 mg kg^{-1} min^{-1} i.v.) on the response to three Ca^{2+} infusions into the LADCA of E_{es} in the distribution areas of the LADCA and the LCXCA of non-stunned (top panels) and stunned (bottom panels) in vivo porcine myocardium. C = control; $^{*}P < 0.05$ Ca^{2+} vs. control (only for baseline); $^{\dagger}P < 0.05$ EMD 57033 vs. baseline (only for control) or stunning + propranolol + EMD 57033 vs. stunning + propranolol (only for control); $^{\ddagger}P < 0.05$ Ca^{2+}-response significantly different from Ca^{2+}-response during baseline; $^{\S}P < 0.05$ Ca^{2+}-response significantly different from Ca^{2+}-response during stunning + propranolol.

LVESP–segment area relation (Fig. 3C) reflected by a recovery of A_0 to 26 ± 4 mm^2 ($P < 0.05$). E_{es} was not significantly affected in the tested dose range of Ca^{2+}. The response of the LVESP–segment area relation of the stunned myocardium to the Ca^{2+} infusions was not modified by propranolol (Figs. 3C and 4C). During the subsequent infusion of EMD 57033, E_{es} returned to baseline values, while A_0 recovered partly (Table 2). Importantly,

Table 2
Effect of EMD 57033 on control values of E_{es} and A_o of propranolol-treated pigs with non-stunned myocardium and of pigs with stunned myocardium

	Propranolol-treated pigs with non-stunned myocardium ($n = 8$)		Pigs with stunned myocardium ($n = 7$)			
	Baseline	EMD 57033	Baseline	Stunning	Stunning + propranolol	Stunning + propranolol + EMD 57033
$E_{es,\ LADCA}$ (mmHg mm^{-2})	6.9 ± 0.9	18.3 ± 2.8^{a}	6.6 ± 1.2	5.3 ± 0.6	5.0 ± 0.9	6.8 ± 1.8^{b}
$A_{0,\ LADCA}$ (mm^2)	27 ± 3	30 ± 1	24 ± 4	44 ± 2^{c}	41 ± 3	33 ± 3^{b}
$E_{es,\ LCXCA}$ (mmHg mm^{-2})	8.1 ± 1.2	18.0 ± 3.5^{a}	6.5 ± 0.8	7.1 ± 1.2	6.6 ± 1.4	11.8 ± 2.0^{b}
$A_{0,\ LCXCA}$ (mm^2)	30 ± 3	32 ± 2	27 ± 3	28 ± 2	30 ± 2	32 ± 1

E_{es}, end-systolic elastance; A_0, area intercept at zero pressure of the LVESP-segment area relation. Values are mean ± S.E.M.
There were no statistical differences between stunning + propranolol and stunning.
[a] $P < 0.05$ EMD 57033 vs. baseline.
[b] $P < 0.05$ stunning + propranolol + EMD 57033 vs. stunning + propranolol.
[c] $P < 0.05$ stunning vs. baseline.

the response of E_{es} to the Ca^{2+} infusions was restored to control levels (Fig. 4C).

In the adjacent myocardium perfused by the LCXCA, neither E_{es} nor A_0 responded to the Ca^{2+} infusions into the LADCA before or after stunning the perfusion territory of the LADCA (Fig. 4D), while E_{es} almost doubled during intravenous infusion of EMD 57033 (Table 2).

4. Discussion

4.1. In vivo assessment of myocardial responsiveness to Ca^{2+}

In analogy to the time-varying elastance concept (Suga, 1990), we employed the slope E_{es} of the LVESP–segment area relation to obtain a load-insensitive index of regional contractile function. A basic assumption for the determination of E_{es} is that during the preload reduction, myocardial contractility (end-systolic stiffness of the myocardial tissue) does not change. For this reason, the preload reductions can only be studied during a period lasting no longer than 6–7 s (Aversano et al., 1986) to avoid hypotension-induced reflex-mediated autonomic nervous system activity alterations. Consequently, the range over which the LVESP–segment area can be determined is limited. At a given level of contractility, the LVESP–segment area relation reflects that particular contractile state independent of whether the relation is constructed via alterations in pre- or afterload. In other words, LVESP and end-systolic area are not independent but interrelated and are determined by the contractile state of the myocardium. Although alterations in diastolic function can modulate the diastolic elastance (and therefore, the left ventricular end-diastolic pressure–segment area relation), this should not affect the position of the LVESP–segment area relation, provided the contractility is unchanged. However, we have previously noted that alterations in diastolic function (i.e. decrease in end-diastolic segment length) can produce translations in the LVESP–segment length relation. Thus, atrial pacing (which produces a decrease in end-diastolic segment length) also produces a leftward translation of the LVESP–segment length relation with no change in E_{es} (Krams et al., 1993). Similarly, the Ca^{2+}-infusions in the present study also produced a leftward translation of the LVESP–segment area relation. These observations are difficult to explain but may be due to methodological limitations of the LVESP–regional segment area relations, which do not take into account the local left ventricular curvature (and hence, the radius) and wall thickness. For instance, at smaller segment areas, the wall thickness increases and radius decreases, which causes a decrease in regional wall stress that is larger than the decrease in left ventricular pressure. Consequently, it is possible that stress–strain relations would not show such a leftward shift.

Intracoronary Ca^{2+} infusions were employed to evaluate the myocardial responsiveness to added Ca^{2+}. As pointed out by Heusch et al. (1996), in in vivo experiments, the intracoronary infusion rates cannot be directly translated into myocardial intracellular Ca^{2+} concentrations. We chose to present the response of E_{es} as a function of Ca^{2+} infusion rates rather than added Ca^{2+} concentrations in the blood. Using added blood concentrations would have yielded identical conclusions, because firstly, coronary blood flows were very similar under control conditions and following stunning, while in the presence of EMD 57033, blood flows were higher. These higher blood flows would result in lower Ca^{2+} concentrations and, hence, the effect of EMD 57033 on Ca^{2+}-responsiveness may have been slightly underestimated. Another potential concern is that intracoronary Ca^{2+} infusions may recruit different amounts of cytosolic Ca^{2+} (Ca^{2+}-induced Ca^{2+} release) in non-stunned myocardium under control conditions and in the presence of EMD 57033 or in stunned myocardium. However, in vitro studies have shown that EMD 57033 increases contractile force (Lues et al., 1993) without an effect on Ca^{2+} transients, indicating that the compound does not modulate activator Ca^{2+}, sarcoplasmic reticulum function or Ca^{2+}-induced Ca^{2+} release (Ferroni et al., 1991; White et al., 1993). Also, several groups of authors have shown that the capacity of the sarcoplasmic reticulum for Ca^{2+} uptake and Ca^{2+} release is preserved in stunned myocardium (Kaplan et al., 1992; Lamers et al., 1993; Duncker et al., 1998; Bolli and Marban, 1999), and that, consequently, the Ca^{2+}-transients in stunned myocardium are not different from those in normal trabeculae (Carrozza et al., 1992; Gao et al., 1995). Moreover, increases in the $[Ca^{2+}]_{out}$ produced identical increases in peak-systolic $[Ca^{2+}]_{in}$ in stunned and normal myocardial trabeculae (Gao et al., 1995), lending further support to the concept that Ca^{2+}-induced Ca^{2+} release and sarcoplasmic reticulum function are unperturbed in stunned myocardium. Similar to other in vivo studies (Buffington and Rothfield, 1995; Heusch et al., 1996; Crystal and Zhou, 1999), maximal Ca^{2+}-activated force could not be determined, because of the occurrence of arrhythmias and contracture-like phenomena (evidenced by marked reductions in end-diastolic segment length) and post-Ca^{2+}-infusion loss of regional contractile function at higher concentrations of intracoronary Ca^{2+}, which we observed in pilot experiments. In the present study, we used slightly lower Ca^{2+} concentrations as compared to previous studies (Ito et al., 1987; Buffington and Rothfield, 1995; Heusch et al., 1996; Crystal and Zhou, 1999) to facilitate detection of the putative Ca^{2+}-sensitizing properties of EMD 57033. Nevertheless, we observed in the normal myocardium that the intracoronary Ca^{2+}-infusions, in the dose-range tested, elicited relative increases in left ventricular dP/dt_{max} (10–20%) and segment shortening

(13%, not presented) that were similar to the 15% increases in wall thickening (Ito et al., 1987; Buffington and Rothfield, 1995), external work (Heusch et al., 1996) or segment shortening (Crystal and Zhou, 1999) and the 10–15% increases in left ventricular dP/dt_{max} (Ito et al., 1987; Heusch et al., 1996; Buffington and Rothfield, 1995; Crystal and Zhou, 1999) found in earlier studies.

4.2. Rationale for the dose of EMD 57033

To allow evaluation of the effect of EMD 57033 on the contractile response to the three 5-min intracoronary Ca^{2+} infusions, a steady state of hemodynamics and contractile function is mandatory during infusion of EMD 57033. In a previous study (De Zeeuw et al., 2000), we observed that systolic shortening, as well as E_{es}, continued to increase throughout the 60 min infusion period of EMD 57033 at a rate of 0.2 mg kg^{-1} min^{-1}. Consequently, we selected a lower dose (0.1 mg kg^{-1} min^{-1}) and observed in the initial experiments that this dose was still effective as E_{es} in the myocardium perfused by the LCXCA increased from 8.1 ± 1.2 to 18.0 ± 3.5 mmHg mm^{-2} after 30 min of infusion, but did not further increase during the subsequent Ca^{2+} infusions into the LADCA (Fig. 4), while the intravenous EMD 57033 infusion was continued. The latter observation precludes the necessity of a control group, in which Ca^{2+} infusions are replaced by a vehicle to exclude the possibility that increases in E_{es} during the intracoronary Ca^{2+} infusions were caused by a progressive increase in contractility secondary to the continuous infusion of EMD 57033.

4.3. Effect of EMD 57033 on Ca^{2+}-responsiveness of non-stunned myocardium

Calcium-sensitizing agents are a heterogeneous class of drugs (Haikala and Linden, 1995; Endoh, 1998; Teramura and Yamakado, 1998). Thus, several of these agents have been shown to increase Ca^{2+}-sensitivity of the myofilaments in vitro by modifying the interaction between Ca^{2+} and Troponin C (e.g. sulmazole, levosimendan, pimobendan, MCI-154 and EMD 60263), by modifying the interaction between the various components of the thin filaments (e.g. pimobendan and MCI 154) or by altering actin–myosin crossbridge kinetics (EMD 57033). In addition to the Ca^{2+}-sensitizing properties, most of these agents also produce considerable PDE-III inhibitory activity (sulmazole, pimobendan, levosimendan and MCI-154). The exact mechanism by which EMD 57033 increases the Ca^{2+} sensitivity is still debated (Lee and Allen, 1997). Solaro et al. (1993) proposed that EMD 57033 principally acts at the actin–myosin site, where the compound reverses the inhibition of actin–myosin interactions by troponin–tropomyosin and may also promote transition of cross-bridges from weak to strong force-generating states. On the other hand, Pan and Johnson (1996) observed in an in vitro model of pure recombinant human cardiac TnC that EMD 57033 binds to the Ca^{2+}/Mg^{2+} sites of TnC.

In the present study, 0.1 mg kg^{-1} min^{-1} EMD 57033 not only increased E_{es} but also enhanced the myocardial response to added Ca^{2+}. Thus, while E_{es} doubled (from 6.9 ± 0.9 to 13.8 ± 3.8 mmHg mm^{-2}) during the intracoronary Ca^{2+} infusions in the absence of EMD 57033, E_{es} tripled (from 18.3 ± 2.8 to 55.7 ± 10.0 mmHg mm^{-2}) during the Ca^{2+} infusions in the presence of EMD 57033, despite the higher control value during infusion of EMD 57033.

Several lines of evidence suggest that in the present in vivo study, the EMD 57033-induced systolic actions are principally the result of Ca^{2+}-sensitization, with a negligible contribution of PDE-III inhibition. In in vitro studies, varying dosages of EMD 57033 have been used. For instance, Grandis et al. (1995) found in Langendorff perfused rat hearts that a dose of only 2 μM could already increase contractility without a significant change in MVO_2. Furthermore, Korbmacher et al. (1994) used 30 μM EMD 57033 in isolated rabbit hearts and showed that at that concentration, EMD 57033 exerts its effect by both Ca^{2+}-sensitizing and PDE-III inhibitory properties. On the other hand, White et al. (1993), who used EMD 57033 in a dose range of 0.1–20 μM in isolated ferret cardiac muscle, reported that EMD 57033 acts predominantly by increasing myofilament Ca^{2+} sensitivity. Taken together, these in vitro studies suggest that PDE-III inhibition occurs principally at concentrations of EMD 57033 in excess of 20 μM. In our previous study (De Zeeuw et al., 2000), with EMD 57033 (0.2 mg kg^{-1} min^{-1}), plasma levels increased time-dependently to 8, 12, 15 and 17 μM at 15, 30, 45 and 60 min infusion, respectively, which is below the in vitro PDE-III inhibiting threshold concentration of 20 μM. Since in the present study we infused 0.1 mg kg^{-1} min^{-1}, this dose would therefore also not be expected to produce PDE-III inhibition. This is supported by the finding that the increases in both E_{es} and left ventricular dP/dt_{max} produced by 0.2 mg kg^{-1} min^{-1} EMD 57033 (maximal plasma levels 17 μM) in normal myocardium were not altered by propranolol, suggesting minimal contribution of PDE-III inhibition to the positive inotropic actions of EMD 57033. In contrast, we have previously shown that the same dose of propranolol virtually abolished the inotropic actions of the phosphodiesterase inhibitor/Ca^{2+}-sensitizer pimobendan (Duncker et al., 1987). Moreover, the duration of both global and regional left ventricular systole were not altered by EMD 57033 in the study by De Zeeuw et al. (2000), independent of the presence of propranolol, indicating that a positive lusitropic effect of PDE-III inhibition was also unlikely. Taken together, the in vivo observation of an enhancement by EMD 57033 of the Ca^{2+}-induced increase in E_{es} is highly consistent with the in vitro observation that EMD 57033

increases myocardial contractile force via an increase in myofilament Ca^{2+} responsiveness.

4.4. Effect of EMD 57033 on Ca^{2+}-responsiveness of stunned myocardium

Myocardial stunning was characterized by a trend towards a decrease in E_{es} and a marked rightward shift of the LVESP–segment area relation. At first glance, this appears to be a surprising finding as most studies (including some from our own laboratory) have reported both a decrease in E_{es} and a rightward shift, although some studies have also reported a rightward shift as the most prominent feature of stunning (Stahl et al., 1986; Krams et al., 1994). The reason for the differences is unclear, but may be related to the range of pressures over which the LVESP–segment area relation was constructed. It is well known that the LVESP–segment length relation may be curvilinear and that the curvilinearity increases at higher end-systolic pressures that are obtained when increases in afterload are used (Miller, 1994). For this reason, we used preload reductions to yield a pressure range of 40 mmHg over which good linearity was observed. A rightward shift of the LVESP–segment area relation is compatible with a decrease of elastic-restoring forces, probably induced by alterations in structural non-contractile elements, such as the extracellular collagen matrix and/or the cytoskeleton. However, as outlined under Section 4.1, the use of LVESP–segment area relations, rather than of left ventricular end-systolic stress–strain relations, may also have contributed to the marked rightward shift.

It is now generally accepted that the mechanism underlying myocardial stunning does not involve a decreased Ca^{2+} availability (Kaplan et al., 1992; Lamers et al., 1993; Gao et al., 1995; Bolli and Marban, 1999), but a decreased responsiveness of the myofilaments to Ca^{2+} (Gao et al., 1995; Bolli and Marban, 1999). However, experimental support for this hypothesis is derived from studies in isolated muscle preparations, whereas evidence obtained in in vivo experiments is lacking. Thus, several groups of investigators have shown that in stunned myocardium, the response of systolic wall thickening (Ito et al., 1987), segment shortening (Buffington and Rothfield, 1995) or external work (Heusch et al., 1996) to intracoronary Ca^{2+} infusions is not impaired. In two of these studies, Ca^{2+} infusions were used that produced maximal levels of systolic shortening (Buffington and Rothfield, 1995) and external work (Heusch et al., 1996) during control conditions, but whether these concentrations also resulted in maximum responses in stunned myocardium was not determined. Only Ito et al. (1987) used Ca^{2+} doses that resulted in maximum responses of systolic shortening in both normal and stunned myocardium, and demonstrated an unperturbed maximum wall thickening in response to Ca^{2+} following stunning. Consistent with previous studies, we observed a similar Ca^{2+}-induced increase in area reduction in normal and stunned myocardium. In contrast, however, in the dose-range tested, the Ca^{2+}-induced increases in E_{es} were depressed following stunning. These findings can be explained by a rightward shift of the $[Ca^{2+}]$-contractile force relations, i.e. a decrease in Ca^{2+} sensitivity. A decrease in Ca^{2+} sensitivity is supported by the study of Hofmann et al. (1993) who showed a rightward shift of the $[Ca^{2+}]$-force relation with a maintained maximum Ca^{2+}-activated force. In addition, a decrease in maximum Ca^{2+}-activated force may also have contributed to the decreased Ca^{2+} responsiveness (Gao et al., 1995).

An interesting observation in the present study was that the increase in E_{es} produced by EMD 57033 in stunned myocardium was less than in non-stunned myocardium. In our previous study, we also observed that at lower concentrations (12 μM), the effect of EMD 57033 on E_{es} was more pronounced in non-stunned than in stunned myocardium (De Zeeuw et al., 2000), while at higher concentrations (17 μM), the E_{es} of non-stunned and stunned myocardium were no longer different. However, interpretation of these observations is difficult, because stunned myocardium perfused by the LADCA was compared to non-stunned myocardium perfused by the LCXCA, and hence, regional differences in contractile responses cannot be excluded. In the present study, we directly compared stunned myocardium to non-stunned myocardium of the same distribution area, which points, indeed, towards a reduced sensitivity of stunned myocardium to the actions of EMD 57033. This is also supported by observations by Korbmacher et al. (1997) who found that non-stunned isolated rabbit hearts were also more sensitive to the Ca^{2+}-sensitizing effects of EMD 60263 than stunned hearts.

Both the response of E_{es} to EMD 57033 as well as the response of E_{es} to the Ca^{2+} infusions in the presence of EMD 57033, were less in stunned than in non-stunned LADCA perfused myocardium. However, infusion of 0.1 mg kg^{-1} min^{-1} of EMD 57033 not only increased E_{es} of stunned myocardium (from 5.0 ± 0.9 vs. 6.9 ± 0.9 mmHg mm^{-2}), but also restored its response to the Ca^{2+} infusions to baseline levels. These findings are consistent with a decreased myofilament Ca^{2+} responsiveness of stunned myocardium, and a restoration of myofilament Ca^{2+} responsiveness by EMD 57033. It is very well possible that the use of a higher dose of EMD 57033 in stunned myocardium that would have resulted in an increase in E_{es} comparable to the level of E_{es} produced by 0.1 mg kg^{-1} min^{-1} in non-stunned myocardium (i.e. 18.3 ± 2.8 mmHg mm^{-2}) would also result in comparable responses to intracoronary Ca^{2+}.

4.5. Conclusions

EMD 57033 enhanced the myocardial responsiveness to intracoronary added Ca^{2+} in non-stunned and stunned

myocardium which supports the concept, based on in vitro observations, that EMD 57033 increases myocardial contractility via an increase in myofilament Ca^{2+}-responsiveness, and that myofilament Ca^{2+}-responsiveness of stunned myocardium is decreased, but can be restored by EMD 57033.

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