Early Exercise Training Normalizes Myofilament Function and Attenuates Left Ventricular Pump Dysfunction in Mice With a Large Myocardial Infarction


Abstract—The extent and mechanism of the cardiac benefit of early exercise training following myocardial infarction (MI) is incompletely understood, but may involve blunting of abnormalities in Ca\textsuperscript{2+}-handling and myofilament function. Consequently, we investigated the effects of 8-weeks of voluntary exercise, started early after a large MI, on left ventricular (LV) remodeling and dysfunction in the mouse. Exercise had no effect on survival, MI size or LV dimensions, but improved LV fractional shortening from 8±1 to 12±1%, and LVdP/dt\textsubscript{max} from 5295±207 to 5794±207 mm Hg/s (both \(P<0.05\)), and reduced pulmonary congestion. These global effects of exercise were associated with normalization of the MI-induced increase in myofilament Ca\textsuperscript{2+}-sensitivity (\(\Delta pC_{a_{50}}=0.037\)). This effect of exercise was PKA-mediated and likely because of improved \(\beta_1\)-adrenergic signaling, as suggested by the increased \(\beta_1\)-adrenoceptor protein (48%) and cAMP levels (36%; all \(P<0.05\)). Exercise prevented the MI-induced decreased maximum force generating capacity of skinned cardiomyocytes (\(F_{\text{max}}\) increased from 14.3±0.7 to 18.3±0.8 kN/m\(^2\) \(P<0.05\)), which was associated with enhanced shortening of unloaded intact cardiomyocytes (from 4.1±0.3 to 7.0±0.6% \(P<0.05\)). Furthermore, exercise reduced diastolic Ca\textsuperscript{2+}-concentrations (by ~30%, \(P<0.05\)) despite the unchanged SERCA2a and PLB expression and PLB phosphorylation status. Importantly, exercise had no effect on Ca\textsuperscript{2+}-transient amplitude, indicating that the improved LV and cardiomyocyte shortening were principally because of improved myofilament function. In conclusion, early exercise in mice after a large MI has no effect on LV remodeling, but attenuates global LV dysfunction. The latter can be explained by the exercise-induced improvement of myofilament function. (Circ Res. 2007;100:1079-1088.)

Key Words: cardiac function ■ cardiomyocytes ■ exercise training ■ heart failure

Left ventricular (LV) remodeling after myocardial infarction (MI) is a compensatory mechanism that serves to restore LV pump function. Despite the apparent appropriateness of LV remodeling to maintain cardiac pump function early after MI, remodeling is an independent risk factor for the development of congestive heart failure.1 The mechanism underlying the progression from LV remodeling to overt heart failure remains incompletely understood, but recent evidence indicates that abnormalities in myofilament function and Ca\textsuperscript{2+}-handling contribute to the LV dysfunction in the porcine heart, early after MI.2 In contrast to pathological LV remodeling after MI, LV remodeling produced by regular dynamic exercise is associated with a decreased risk for coronary artery disease and heart failure.3 Exercise training is associated with an increased myocardial perfusion capacity and with normal or even increased contractile function in the normal heart.4,5 There is also clinical evidence that exercise after MI has a beneficial effect on disease progression and survival.6,7 For example, physical conditioning in patients with LV dysfunction results in an increased exercise capacity which has been ascribed, at least in part, to skeletal muscle adaptations.8

The effects of exercise on LV remodeling and function are still incompletely understood, as several studies in humans reported contradictory effects of training on LV remodeling after a MI.9-18 Careful inspection of these studies suggests that after a small MI, exercise has no detrimental effect11,13 or even improves15,17,18 LV geometry and function, independent of whether exercise was started late, ie, ≈1 year,17,18 or early, ie, <2 months,11,13,15 after MI. In contrast, in patients with a...
large MI, exercise had either no,14 or a beneficial18 effect on ejection fraction (EF) and LV volumes but only when started late after MI. However, when exercise after a large MI is started at a time when LV remodeling is still ongoing (<3 to 4 months after MI), the majority of studies reported that exercise has either no,11–13 or even a detrimental9,10 effect on LV volume and EF.

Similar to these clinical studies, studies in rats indicate that exercise started late (>3 weeks) after a moderate to large MI, encompassing 35% to 50% of LV mass, at a time when infarct healing is complete, does not aggravate,19,20 or even blunts21–23 LV dilation and hypertrophy. In contrast, when starting <1 week after a moderate to large MI,24–27 exercise resulted in variable outcomes with beneficial,28 no,24,27 or detrimental25,26 effects on LV remodeling. These rodent studies lend further support to the concern that early exercise may have detrimental effects on LV remodeling after a large MI, although interpretation is hampered by the fact that late exercise studies in rats principally used treadmill running.18,19,21,22 whereas early exercise studies predominantly used swimming.23–25,27,28 This is important because the exercise responses to swimming are markedly different from those to treadmill running.29,30

In light of these observations, the first aim of the present study was to assess the effects of exercise by voluntary treadmill running, started within 24 hour after a large MI, on LV remodeling and dysfunction in the mouse. The results indicated that exercise attenuated the MI-induced LV dysfunction, without a detrimental effect on LV remodeling. Consequently, we tested the hypothesis that exercise early after a large MI is able to reverse the MI-induced abnormalities in β1-adrenergic receptor and Ca2+-handling protein expression, phosphorylation status of contractile proteins, Ca2+-handling and myofilament function, within the noninfarcted remodeled myocardium.

**Materials and Methods**

For detailed description see the expanded Materials and Methods in the online data supplement available at http://circres.ahajournals.org. Experiments complied with The Guide for Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication No. 86-23, Revised 1996), and were approved by the Erasmus MC Animal Care Committee.

**Experimental Groups**

147 C57Bl/6J mice of either sex (~12-weeks old) entered the study and were randomly assigned to one of four experimental groups. Sham-operated mice (SH) and mice with MI were housed sedentary (SHSED, MISED) or subjected to voluntary exercise training (SHEX, MIEX) for 8-weeks.

**Experimental Procedures**

MI was produced by permanent ligation of the left-antiorior-descending-coronary-artery (LAD).31,32 Eight weeks after entering the study, mice were anesthetized and instrumented for hemodynamic measurements.32 M-mode LV echocardiography was performed and LV diameters at end-diastole (LVd) and end-systole (LVs) were measured, and fractional-shortening (FS) calculated. Pressure-diameter relations were obtained from M-mode images synchronized with LV-pressure by simultaneous ECG recording.32

**Tissue Analysis**

Right (RVW) and left (LVW) ventricular weight, tibial-length (TL) and lung-fluid weight were determined in each animal. Masson’s trichrome-staining was used for analysis of LV collagen volume fraction and cardiomyocyte cross-sectional area (CSA) (n=8/group). Endocardial and epicardial infarct-circumference, infarct-thickness, and infarct-surface-area were determined.35 In 8 mice, infarct-size was determined 24 hour after LAD ligation. Skeletal muscle samples were obtained to determine maximal citrate-synthase (CS) activity in 12 SHEX and 12 SHSED.35

**Force Measurements in Single Permeabilized Cardiomyocytes**

Isometric force was measured in single permeabilized cardiomyocytes of 5 mice per group at different [Ca2+]i and a sarcomere length of 2.2 μm.2 Rate-of-force redevelopment (Ktr) was determined at pCa values ranging from 4.5 to 5.8 using the release-restretch method.2,34 After obtaining a complete force–pCa series, myocytes were incubated in relaxing solution containing the exogenous catalytic subunit of protein-kinase A (PKA) and a second force–pCa series was obtained.2,34 Force-pCa relations were fit to the Hill equation.2,34

**Myosin-Heavy-Chain Composition**

Myosin-heavy-chain (MHC) isoform composition was analyzed by 1-dimensional SDS-PAGE.35

**Myofilament Proteins Phosphorylation Status**

LV samples were separated on gradient gels and stained with Pro-Q Diamond phosphoprotein gel stain in conjunction with SPROY Ruby staining. The phosphorylation signals for myofilament proteins were normalized to the intensities of the SPROY Ruby stained myosin binding protein-C (MyBP-C) bands and analyzed.

**β1-Adrenergic Signaling**

cAMP levels were measured in LV samples, homogenized in 100 μL frozen 0.1 mol/L HCl, using an enzyme immunoassay kit.2 PKA levels were measured in LV samples, homogenized in 100 μL frozen PKA extraction buffer, using PepTag Assay kit.2

**Western Immunoblotting**

LV samples were homogenized and protein concentrations were determined. Proteins were separated by SDS-PAGE and blots were stained reversibly with Ponceau Red, and incubated overnight at 4°C with diluted primary antibodies. Signals were visualized using Supersignal West Femto Maximum Sensitivity Substrate and Hyperfilm ECL and quantified.

**Contractile Properties of Intact Cardiomyocytes**

Single LV cardiomyocytes were obtained from the noninfarcted part of the LV in 11 additional mice (6 MISED and 5 MIEX) by enzymatic dissociation. Unloaded cell shortening and intracellular Ca2+–concentrations [Ca2+1], were studied using field stimulation and ruptured patch clamp recording techniques.36

**Statistics**

Data were analyzed using two-way ANOVA, followed by post hoc testing with Student-Newman-Keuls, or using unpaired t-testing, as appropriate. Survival was analyzed by Kaplan-Meier method and log-rank (Mantel-Cox) test. Significance was accepted when P<0.05. Data are means±SEM.

**Results**

**Exercise and Survival**

MIEX initially ran shorter distances per day compared with SHEX (Figure 1), but total distance over the 8-week period and hence daily distance was similar in SHEX (5.9±0.2 km/d) and
Global LV Function
MI resulted in lower LV systolic pressure, LVdP/dt30, fractional shortening, LVdP/dtmin and increased τ, but had no apparent effect on LV end-diastolic pressure (Table). Exercise had minimal effects on LV systolic and diastolic function in Sham mice, but increased both LVdP/dt90 and FS after MI.

MI resulted in a marked rightward shift as well as a narrowing of the LV pressure-diameter relation, indicating LV dilation and depressed FS (Figure 3D). Exercise caused a small leftward shift in Sham and MI animals (both P<0.02). Lung-fluid weight and RVW/TL were increased after MI, indicative of pulmonary congestion and RV hypertrophy (Figure 3E and 3F), which were abolished by exercise.

Force Development in Single Permeabilized Cardiomyocytes
Passive force was similar in SHSED (3.0±0.4 kN/m²) and MIEX (2.9±0.3 kN/m²), but maximal isometric force (Fmax) was significantly lower in MIEX than in SHSED (Figure 4A). The normalized force-pCa curves also showed a leftward shift in MI animals, indicating greater Ca²⁺-sensitivity in MIEX than in SHSED, which was accompanied by an increased steepness (nHill) of the force-pCa curves in MI compared with Sham (2.4±0.1 versus 2.6±0.1 respectively, P=0.05). Treatment with the catalytic subunit of PKA decreased pCa₅₀ in both MIEX and SHSED, reflecting a PKA-induced decrease in myofilament Ca²⁺-sensitivity. Importantly, after PKA the Ca²⁺-sensitivity was no longer different between MIEX and SHSED (Figure 4), suggesting that loss of PKA-mediated myofilament protein phosphorylation contributed to the increased myofilament Ca²⁺-sensitivity after MI. PKA had no effect on Fmax in MIEX and SHSED (not shown).

Exercise had no effect on passive force of cardiomyocytes from either Sham or MI mice (not shown). Exercise had also no effect on contractile properties of cardiomyocytes from either Sham or MI mice (not shown). Exercise had also no effect on contractile properties of cardiomyocytes from either Sham or MI mice (not shown). Exercise had also no effect on contractile properties of cardiomyocytes from either Sham or MI mice (not shown). Exercise had also no effect on contractile properties of cardiomyocytes from either Sham or MI mice (not shown). Exercise had also no effect on contractile properties of cardiomyocytes from either Sham or MI mice (not shown). Exercise had also no effect on contractile properties of cardiomyocytes from either Sham or MI mice (not shown).

LV Remodeling
Twenty-four hours after LAD ligation, 88±2% of the area-at-risk had become infarcted, corresponding to 43±3% of the LV. This resulted in marked LV dilation and hypertrophy, reflected in the increased LV diameter, relative LVW (LVW/TL), and increased cardiomyocyte-CSA and LV collagen volume fraction within the remote myocardium 8 weeks later (Figure 2 and Figure 3A through 3C).

Exercise had no significant effect on cardiomyocyte-CSA and LVW/TL in either Sham or MI mice. However, exercise tended to decrease LV end-diastolic diameter and significantly reduced LV collagen content in both Sham and MI mice (Figure 3).
protein in MI_{SED} to 6.1±0.8 pmol/mg protein in MI_{EX} (P<0.05).

**Western Immunoblotting**

Protein level of the β₁-adrenergic receptor decreased after MI, but did not change in SH_{EX} mice. The decrease was not accompanied by significant changes in GRK2 and G_{i-3} expressions (Figure 6). Protein levels of SERCA2a decreased in the remodeled myocardium after MI, but were not altered in SH_{EX} mice. PLB levels were maintained in both MI_{SED} and SH_{EX}. PLB phosphorylation at the Ser16 site did not change in both MI and exercise mice, whereas phosphorylation at the Thr17 site was decreased in both groups. Na⁺/Ca²⁺-exchanger levels did not change in MI_{SED} and SH_{EX} mice.

Exercise after MI increased β₁-adrenergic receptor levels and Na⁺/Ca²⁺-exchanger levels, but had minimal effects on the expression of GRK2, G_{i-3}, SERCA2a, and PLB and PLB phosphorylation at both Ser16 and Thr17 sites (Figure 5).

**Contractile Properties of Isolated Intact Cardiomyocytes**

To further investigate the mechanism by which exercise improved LV function in MI mice, we performed additional experiments in enzymatically isolated intact cardiomyocytes obtained from MI_{SED} and MI_{EX}. Unloaded cell shortening in MI_{EX} was significantly higher compared with MI_{SED} mice (Figure 7). Preliminary cell shortening data of 3 SH_{SED} mice showed values comparable to MI_{EX} (not shown). Basal [Ca²⁺],
The present study investigated the impact of 8 weeks of voluntary exercise training, started early after a large MI, on LV remodeling and dysfunction in mice at the in vivo, cellular and molecular level. The main findings were that: (1) exercise had no adverse effect on LV dimensions and hypertrophy, while ameliorating LV dysfunction and backward failure; (2) exercise normalized MI-induced myofilament dysfunction, which likely contributed to the exercise-induced improvement in unloaded shortening of isolated intact cardiomyocytes, as the [Ca\(^{2+}\)], transient amplitude was not altered by exercise. In addition, basal [Ca\(^{2+}\)], was reduced by exercise; and (3) exercise likely mediated these effects via increased β1-adrenergic receptor protein and cAMP levels, and Na\(^+\)/Ca\(^{2+}\)-exchanger protein levels.

**Pathophysiology of MI-Induced LV Dysfunction in Mice**

In agreement with previous reports,\(^{31,32,37}\) permanent LAD ligation in mice resulted in LV remodeling, characterized by LV dilation, hypertrophy, and increased collagen deposition in remote noninfarcted myocardium, and resulted in marked LV dysfunction, characterized by decrements in LV pump function (fractional shortening) and decrements in indices of global LV contractility (dP/dt\(_{max}\)) and relaxation (dP/dt\(_{min}\)), which was associated with LV backward failure reflected in pulmonary edema and RV hypertrophy. The mechanism for LV dysfunction after MI remains incompletely understood, but it has been proposed to be the consequence of alterations in LV geometry with no effect on cardiomyocyte function or β1-adrenergic responsiveness.\(^{38}\) Conversely, other investigators reported that alterations in β1-adrenergic signaling\(^{21}\) and Ca\(^{2+}\)-handling\(^{22,23}\) of the remote myocardium also contribute to global LV dysfunction. In agreement with the latter notion, we observed downregulation of β1-adrenergic receptor levels and reductions in SERCA2a after MI, whereas no clear changes were found in protein levels of GRK2, Goαi3, PLB and Na\(^+\)/Ca\(^{2+}\)-exchanger. Unexpectedly, cAMP levels were not depressed in MI mice, which is consistent with the observation that cAMP-dependent PKA mediated phosphorylation of PLB-Ser16 was also not decreased after MI, but suggests that the β1-adrenergic signaling in MI mice is not a simple function of the β1-receptor density. PLB-Thr17 phosphorylation, which is mediated by CaM-kinase II, was attenuated following MI. Reduced phosphorylation of PLB-Thr17 will inhibit SERCA2a function in the mouse heart particularly at higher heart rates.\(^{39}\) The latter could, in conjunction with decreased β1-adrenoceptor and SERCA2a expression, contribute to perturbations in cardiomyocyte Ca\(^{2+}\)-handling in MI mice, particularly during increased activity.

In agreement with observations in swine,\(^{2}\) remodeling of noninfarct myocardium in mice was associated with altered myofilament function, characterized by decreased F\(_{max}\) and increased Ca\(^{2+}\)-sensitivity of tension development in single permeabilized cardiomyocytes. The small increase in Ca\(^{2+}\)-sensitivity after MI was likely the result of the minor reduction of PKA-mediated phosphorylation of TnI and MyBP-C,\(^{2,40}\) as treatment with exogenous PKA abolished the difference in Ca\(^{2+}\)-sensitivity between Sham and MI animals. However, Pro-Q Diamond analysis did not reveal significant decreases in phosphorylation status of myofilament proteins in MI\(_{SED}\) compared with SH\(_{SED}\). It is possible that a small

---

**LV Anatomical and Functional Data**

<table>
<thead>
<tr>
<th></th>
<th>Sedentary</th>
<th>Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anatomical data</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>26 ± 1</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>MI</td>
<td>26 ± 1</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>LV weight, mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>96 ± 4</td>
<td>95 ± 4</td>
</tr>
<tr>
<td>MI</td>
<td>109 ± 4*</td>
<td>110 ± 4*</td>
</tr>
<tr>
<td>Endocardial infarct length, mm</td>
<td>3.8 ± 0.3</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>MI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epicardial infarct length, mm</td>
<td>4.6 ± 0.3</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>MI</td>
<td>0.11 ± 0.01</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>Infarct area, mm²</td>
<td>1.54 ± 0.14</td>
<td>1.59 ± 0.08</td>
</tr>
<tr>
<td>MI</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hemodynamic data</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>537 ± 8</td>
<td>555 ± 12</td>
</tr>
<tr>
<td>MI</td>
<td>520 ± 7</td>
<td>528 ± 9</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>70 ± 2</td>
<td>79 ± 5\†</td>
</tr>
<tr>
<td>MI</td>
<td>69 ± 2</td>
<td>71 ± 1*</td>
</tr>
<tr>
<td>LVSP, mm Hg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>93 ± 2</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>MI</td>
<td>83 ± 2*</td>
<td>82 ± 2*</td>
</tr>
<tr>
<td>LV dP/dt(_{max}), mm Hg/s</td>
<td>7189 ± 239</td>
<td>7669 ± 267</td>
</tr>
<tr>
<td>Sham</td>
<td>5295 ± 207*</td>
<td>5891 ± 204\†</td>
</tr>
<tr>
<td>MI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV dP/dt(_{syst}), mm Hg/s</td>
<td>39 ± 2</td>
<td>47 ± 3\†</td>
</tr>
<tr>
<td>Sham</td>
<td>8 ± 1*</td>
<td>12 ± 1\†</td>
</tr>
<tr>
<td>MI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tau, ms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>7.9 ± 0.5</td>
<td>8.1 ± 1.0</td>
</tr>
<tr>
<td>MI</td>
<td>10.6 ± 0.8*</td>
<td>10.5 ± 1.1*</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>6.4 ± 0.6</td>
<td>4.3 ± 0.9</td>
</tr>
<tr>
<td>MI</td>
<td>7.9 ± 1.0</td>
<td>5.7 ± 0.8</td>
</tr>
</tbody>
</table>

MAP, mean arterial pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure. SH\(_{SED}\) (n=27), MI\(_{SED}\) (n=27), and SH\(_{EX}\) (n=11), MI\(_{EX}\) (n=27), and MI\(_{EX}\) (n=14). *P<0.05 vs corresponding Sham; †P<0.05 vs corresponding Sedentary.

was lower in MI\(_{EX}\) than MI\(_{SED}\), but the [Ca\(^{2+}\)], transient amplitudes were similar in the 2 groups.

**Discussion**

The present study investigated the impact of 8 weeks of voluntary exercise training, started early after a large MI, on...
Figure 4. Absolute (A and C) and normalized (B and D) force-pCa curves and bar charts. The pCa_{50} before and after treatment with exogenous PKA (E and F). The panels (A, B, and E) show the effects of MI in SED mice. The panels (C, D, and F) show the effects of EX in SH and MI. Myofilament protein phosphorylation of MyBP-C (G), TnI (H), MLC-2 (I), and total PKA (J) in LV remote myocardium. *P<0.05 vs corresponding SH; †P<0.05 vs corresponding SED; ‡P<0.05 after PKA vs before PKA.
LV of MI_{SED} (5.41±2.39 a.u.) compared with SH_{SED} (0.10±0.09 a.u.; \(P=0.016\)), correlating well with lung-fluid weight (\(R^2=0.55; P=0.014\)). An explanation for this unexpected lack of MHC-isofrom shift is not readily found, but it should be noted that our observations are consistent with the unchanged Ktr after MI.

In conclusion, the present study supports the concept that alterations at the cellular level in remote noninfarcted myocardium contribute to decreased global LV function after MI. Future studies, using catecholamine challenges, are required to determine in greater detail the importance of perturbations in kinase-phosphatase signaling cascades in post-MI remodelled mouse heart.

**Mechanism of Beneficial Effects of Exercise Training After MI**

Rat studies on exercise after MI have reported no changes in parameters of LV function such as LV dP/dt_{max}, fractional shortening, PV-relations or cardiac output, irrespective of whether exercise was started early or late, irrespective of a small or large MI, and irrespective of treadmill or swim training. Nevertheless, exercise was reported to improve cardiomyocyte function. Thus, exercise after healed MI (>3 weeks after MI) attenuates \(\beta\)-MHC expression, and restores cardiomyocyte Ca\(^{2+}\)-handling and Ca\(^{2+}\)-responsiveness, and SERCA2a and Na\(^+/\)Ca\(^{2+}\)-exchanger levels. Furthermore, exercise was reported to blunt cardiomyocyte hypertrophy, and to restore Ca\(^{2+}\)-transients, and SERCA2a and Na\(^+/\)Ca\(^{2+}\)-exchanger expression. Thus, beneficial effects of exercise on LV remote myocardium in rats are clearly observed when exercise is initiated late after MI. To date no study has investigated the effects of exercise started early after MI on \(\beta\)-adrenergic signaling, Ca\(^{2+}\)-handling and myofilament function.

In view of the concern that early exercise may aggravate LV remodeling after a large MI, we investigated the effects of exercise started immediately after MI on LV remodeling and dysfunction in the mouse. The results indicate that in mice, even after a large MI (comprising \(\approx43\%\) of LV mass), 8 weeks of voluntary exercise does not aggravate LV remodeling, as relative LV mass and cardiomyocyte size as well as infarct geometry were unchanged, whereas exercise decreased collagen content and actually tended to decrease LV end-diastolic diameter. These observations are in agreement with a recent study by Konhilas et al who reported that 8 weeks of moderate exercise in mice with hypertrophic cardiomyopathy reversed collagen deposition with little effect on cardiac hypertrophy. Interestingly, exercise also reversed expression of hypertrophy markers and components of apoptosis pathways. In view of the minimal effects of exercise on LV remodeling and the pronounced effects on LV dysfunction, we elected to focus on the effects of exercise training on myofilament function and Ca\(^{2+}\)-handling. However, the study by Konhilas et al warrants future studies that include the analysis of hypertrophy and survival signaling pathways in the model of post-MI remodeling.

Exercise attenuated LV dysfunction and ameliorated LV backward failure, which were likely because of improved cardiomyocyte function, as shortening of isolated cardiomyo-
cytes was increased by exercise. Ca\textsuperscript{2+}-transient amplitude remained unaltered, consistent with the lack of effect of exercise on SERCA2a and PLB expression. However, basal (diastolic) calcium concentrations were reduced by exercise after MI. In the absence of changes in SERCA2a and PLB protein levels or phosphorylation, a potential explanation for the reduction in basal [Ca\textsuperscript{2+}], is the increased expression of Na\textsuperscript+/Ca\textsuperscript{2+}-exchanger. However, future studies are needed to determine whether this small increase in Na\textsuperscript+/Ca\textsuperscript{2+}-exchanger protein levels is indeed responsible for the exercise-induced

Figure 6. Immunoblot analyses of \(\beta_1\)-adrenergic signaling and Ca\textsuperscript{2+}-handling proteins of the four groups. Representative Western blots are shown, with summary quantification in bar graphs (n=5 for each bar). Comparisons were made in a pair-wise fashion (normalized within each pair). *P<0.05 vs corresponding white bar.
reduction in diastolic Ca\(^{2+}\) levels, or whether other mechanisms, including increased sarcolemmal Ca\(^{2+}\)-ATPase activity, also contribute.

Importantly, our findings indicate that the improved cell shortening was not because of increased Ca\(^{2+}\)-transient amplitude, suggesting that the exercise-induced normalization of myofilament function was principally responsible for the improved isolated myocyte shortening in vitro and global LV fractional shortening in vivo. Exercise restored myofilament Ca\(^{2+}\)-sensitivity after MI, which was likely mediated via increased \(\beta_1\)-adrenergic signaling as suggested by the increased \(\beta_1\)-adrenoceptor and cAMP levels and by the normalization of the Ca\(^{2+}\)-sensitivity response to PKA. Restoration of \(\beta_1\)-adrenergic signaling also acts to increase MLC-2 phosphorylation via PKA-mediated inhibition of protein phosphatase 1.\(^{49}\) Indeed, MLC-2 phosphorylation was increased by exercise. It is however unlikely that the exercise-induced increase in MLC-2 phosphorylation is involved in either the increased \(F_{\text{max}}\) or reduced Ca\(^{2+}\)-sensitivity in MEX, because phosphorylation of MLC-2 increases myofilament Ca\(^{2+}\)-sensitivity without an effect on \(F_{\text{max}}\).\(^{50,51}\) Future studies are required to further delineate the mechanism underlying the exercise-induced normalization of \(F_{\text{max}}\).

**Clinical Implications**

The present study indicates that exercise training started early after a large MI is beneficial, resulting in improved LV function and molecular phenotype, without adverse effects on LV remodeling. The beneficial effects appear to be the result of improved \(\beta_1\)-adrenergic signaling and myofilament function. Because some of these cellular adaptations to exercise are also observed following chronic \(\beta_1\)-adrenoceptor blockade,\(^{52,53}\) future studies should be aimed at investigating whether combined \(\beta_1\)-adrenoceptor blockade and exercise yield added benefit.

**Sources of Funding**

This study was supported by the Netherlands Heart Foundation grant 2000T038 (D.J.D), the Netherlands Organisation for Scientific Research (VENI grant 2002; J.V.D.V; N.M.B), and Fund for Scientific Research Flanders (G.0166.03N; K.S).

**Disclosures**

None.

**References**

mal remodeling in patients with reduced left ventricular function after myocardial infarc- 
13. Otsuka Y, Takaki H, Okano Y, Sato T, Aihara N, Matsumoto T, Yasumura Y, Morii I, Goto Y. Exercise training without ventricular remodeling in patients with moderate to severe left ventricular dysfunc-
infarction patients with left ventricular dysfunction: results of the Exercise in Left Ventricular Dysfunction (ELVD) trial. Circulation. 1997;96: 
1790–1797.
19. Musch TJ, Moore RL, Leathers DJ, Bruno A, Zelis R. Endurance training in rats with chronic heart failure induced by myocardial infarction. Cir-
21. Orenstein TL, Parker TG, Butany J, Goodman JM, Dawood F, Wen WH, Lee E, Martino T, McLaughlin PR, Liu PP. Favorable left ventricu-
23. Wisloff U, Loennechen JP, Currie S, Smith GL, Ellingsen O. Aerobic exercise reduces cardiomyocyte hypertrophy and increases contractility, 
tissue tension development and its calcium sensitivity in skinned myocyte-sized preparations from different regions of the human heart. Cardiovasc Res. 
27. Antoons G, Mubagwa K, Nevelsteen I, Sipido KR. Mechanisms underlying the frequency dependence of contraction and [Ca2+]i-
31. Strang KT, Sweitzer NK, Greaser ML, Moss RL. Beta-adrenergic receptor stimulation increases unloaded shortening velocity of skinned single 
32. Wang J, Liu X, Senetz E, Takeda N, Dhalla NS. Increased expression of protein kinase C isoforms in heart failure because of myocardial 
Murphy AM, van Eyk JE, Tesi C, Poggesi C, van der Velden J, Stienen GJM. Impaired diastolic function after exchange of endogenous troponin 
34. Burkart EM, Sumaneda MP, Kobayashi T, Nii M, Martin AF, Homsher E, Solaro RJ. Phosphorylation or glutamic acid substitution at protein 
35. Sumaneda MP, Pyle WG, Kobayashi T, de Tombe PP, Solaro RJ. Identification of a functionally critical protein kinase C phosphorylation 
36. Hashimoto T, Kambara N, Nohara R, Yazawa M, Taguchi S. Expression of MHC-beta and MCT1 in cardiac muscle after exercise training in 
37. Musch TJ, Moore RL, Smaldone PG, Riedy M, Zelis R. Cardiac adap-
38. Zhang LQ, Zhang XQ, Ng YC, Rothblum LI, Musch TJ, Moore RL, Cheung JY. Sprint training normalizes Ca2+-transients and SR function in 
39. Konhilas JP, Watson PA, Maas A, Boucek DM, Horn T, Stauffer BL, Luckey SW, Rosenberg P, Leinwand LA. Exercise can prevent and 
reverse the severity of hypertrophic cardiomyopathy. Circ Res. 2006;98: 
540–548.
EG, DePaoli-Roach AA, Robbins J, Hewett JE, Bibb JA, Westfall MV, Kranivas EG, Molkentin JD. PKC-alpha regulates cardiac contractility and propensity 
41. Morano I. Effects of different expression and posttranslational modifi-
cations of myosin light chains on contractility of skinned human cardiac 
42. Rajasheer R, Blunt BC, Hofmann PA. Modulation of myosin phosphate 
targeting subunit and protein phosphatase 1 in the heart. Am J Physiol 
cardiac function and calcium handling protein in postinfarction heart failure 
44. Min YJ, Ding B, Wang JF, Sullivan MF, Morgan JP. Metoprolol attenuates postischemic depressed myocardial function in papillary 
Early Exercise Training Normalizes Myofilament Function and Attenuates Left Ventricular Pump Dysfunction in Mice With a Large Myocardial Infarction

Circ Res. 2007;100:1079-1088; originally published online March 8, 2007; doi: 10.1161/01.RES.0000262655.16373.37
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/100/7/1079

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2007/03/08/01.RES.0000262655.16373.37.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
**Expanded Materials and Methods**

Experiments complied with the “Guide-for-Care-and-Use-of-Laboratory-Animals” of the National Institutes of Health (NIH Publication No. 86-23, Revised 1996), and were approved by the Erasmus MC Animal Care Committee.

*Experimental groups*

A total of 147 C57Bl/6J mice of either sex (10-12 weeks old) entered the study. Animals were randomly assigned to one of four experimental groups. Sham operated mice (Sham) and mice that underwent a MI were subjected to a sedentary life-style (SH\textsubscript{SED}, MI\textsubscript{SED}) or to voluntary exercise training (SH\textsubscript{EX}, MI\textsubscript{EX}). Mice were individually subjected to either sedentary housing or voluntary exercise training (EX) for a period of eight weeks. Treadmills were custom built to allow electronic measurement of the distance run by the mice. Voluntary EX was the training of choice due to its ability to achieve uniform controlled EX in mice and to minimize stress factors, which are present during forced running and particularly during swimming.\(^1\) C57Bl/6J mice were used because they are known to perform excellent in voluntary wheel running.\(^1\)

In an additional group of 8 mice, 24 hours after permanent ligation of the left anterior descending coronary artery (LAD), the LV area at risk (AR) was determined using negative 1% Evans Blue staining administered via the jugular vein. Subsequently the heart was excised, quickly frozen for a few minutes and cut in five slices in the direction from apex to aorta. Infarct area (IA) was determined with negative 2% triphenyltetrazolium chloride (TTC) staining for 5 min at 37°C. After TTC staining, the area of infarction included the anterior wall and apical part of the LV appears pallid, whereas the viable myocardium appears red. The LV was then separated from the RV and each slice was weighed. To calculate IA an AR, both sides of each of the slices were photographed, and the IA and AR for both sides of each section was determined using Sigma Scan Pro 5 software. Infarct size was calculated as IA/AR*100%.\(^2\)
**Experimental Procedures**

Mice were weighed, sedated with 4% isoflurane, intubated and ventilated with a mixture of O\textsubscript{2} and N\textsubscript{2}O (1/2, vol/vol) with a pressure controlled ventilator (CWE, SAR-830/P) to which 2.3% isoflurane was added for anesthesia.\textsuperscript{3} The ventilation rate was set at 90 strokes/min, with a peak inspiration pressure of 18 cmH\textsubscript{2}O and a positive end expiration pressure (PEEP) of 4 cmH\textsubscript{2}O. The mice were placed on a heating pad to maintain body temperature at 37°C. A thoracotomy was performed through the third left intercostal space, after which the lungs were protected with gauze and the pericardium opened. MI was produced, in two groups of animals (MI\textsubscript{SED} and MI\textsubscript{EX}), by permanent ligation of the LAD with a 7-0 silk suture mounted on a tapered needle (B Braun; Aesculap AG&CO. KG, Germany). Subsequently, the chest was closed with a 6-0 silk suture (B Braun; Aesculap AG&CO. KG, Germany) and the skin with suture clips (B Braun; Aesculap AG&CO. KG, Germany) whereupon the mice received 0.05 mg/kg analgesics subcutaneously (Buprenorfinehydrochloride, Reckitt Benekiser Inc., UK) and were allowed to recover.

Eight weeks after entering the study, mice were anesthetized and ventilated as described above. *In vivo* trans-thoracic echocardiography of the LV was performed (ALOKA ProSound SSD-4000; Japan) using a 13-MHz linear array transducer.\textsuperscript{3} At the start of the experiment and after placement of a 1.4F microtipped pressure transducer catheter in the LV (SPR-671, Millar Instruments, Houston, TX; calibrated prior to each experiment with a mercury manometer) echocardiograms were obtained with simultaneous ECG registration. M-mode echocardiograms were captured from short-axis 2D views of the LV at midpapillary level. LV diameters at end diastole and end systole (LV\textsubscript{EDD} and LV\textsubscript{ESD}) were measured from the M-mode images using SigmaScan Pro 5 Image Analysis software (SPSS Inc., Chicago, IL). A blinded observer analyzed three cardiac cycles for each animal in the four experimental groups and fractional shortening was calculated as FS= (LV\textsubscript{EDD}−LV\textsubscript{ESD})/LV\textsubscript{EDD} *100%. Pressure-diameter relations were obtained from M-mode images synchronized with LV pressure by simultaneous recorded ECG and constructed with a program written in MatLab (Mathworks Inc, Natick, MA). Data from four consecutive beats were averaged.
Following echocardiography, the mice were instrumented with a fluid filled polyethylene catheter (PE 10) inserted into the left carotid artery and advanced into the aortic arch for measuring mean aortic pressure and heart rate. A stretched PE 50 catheter was introduced into the left external jugular vein and advanced into the superior caval vein for infusion of Haemaccel (Hoechst Marion Roussel B.V.), to maintain fluid-balance. A 1.4F Millar Instruments pressure transducer catheter was inserted in the right carotid artery and advanced in the LV for measuring LV pressure. Hemodynamic data were recorded and digitized using an on-line four-channel data acquisition program (ATCODAS, Dataq Instruments, Akron, OH), for later analysis with a program written in MatLab. Ten consecutive beats were selected for determination of heart rate, mean aortic pressure, LV systolic pressure, LV end diastolic pressure, the less afterload-sensitive contractility parameter, rate of rise of LV pressure at 30 mmHg (LVdP/dt$_{30}$) and the relaxation parameters the time constant of LV pressure decay (Tau, $\tau$) and the maximum rate of LV pressure decay (LVdP/dt$_{\text{min}}$).

**Tissue Analysis**

At the conclusion of each experiment, the heart was excised and the atria removed after which the right ventricle (RV) and LV including septum were separated. The weight of RV (RVW) and LV (LVW), and tibial length (TL) were determined. Accumulation of lung fluid weight was assessed by measuring the difference between the wet- and dry lung weights, which is an indication for pulmonary edema. In 12 SH$_{\text{SED}}$ and 12 SH$_{\text{EX}}$ animals skeletal muscle samples were obtained for maximal citrate synthase (CS) activity measurements spectrophotometrically on skeletal muscle homogenates (expressed as $\mu$mol.g$^{-1}$ of protein). The LV was cut in two halves along the longitudinal axis. Half of the LV was fixed overnight in freshly prepared 4% paraformaldehyde in PBS and embedded in paraffin for histology, while the other half was quickly frozen in liquid nitrogen for assessment of force measurements in single skinned cardiomyocytes and analysis of the $\beta$1-adrenergic signaling pathway and contractile, Ca$^{2+}$-handling and myofilament proteins.
**Histology.** Four micron thick paraffin LV sections were stained with Masson’s trichrome for analysis of collagen volume fraction and cardiomyocyte cross-sectional area (CSA) measurements within the remote non-infarcted myocardium. Four fields were randomly selected in two sections of eight mice per group and photographed using an Olympus BH 20 microscope (Olympus Corporation, Japan) at a magnification of x400. Within each field, segments representing connective and muscle tissue were identified and manually traced with a digitizing pad and computer image analysis software (Clemex Vision PE 3.5) to calculate the traced area. Collagen volume fraction was calculated in each field as the sum of all connective tissue areas divided by the sum of all connective tissue and muscle areas and averaged for each animal per group. Myocyte CSA was measured by tracing the outline of myocytes showing the nucleus in each field and averaged for each animal in the groups. Finally, the infarct region was demarcated and the area was measured. Endocardial and epicardial infarct circumference were demarcated and the lengths measured. Infarct thickness was measured at the shortest distance between endocardium and epicardium. The operator was blinded to the experimental group during the analysis.

**Force Measurements in Single Permeabilized Cardiomyocytes**

Cardiomyocytes were mechanically isolated from liquid nitrogen frozen LV samples of 5 mice (3 myocytes/animal) per group. Before mechanical isolation, tissue was defrosted in cold relaxing solution (pH 7.0; in mmol/l: free MgCl₂, 1; KCl, 145; EGTA, 2; ATP, 4; imidazole, 10). During the isolation, the tissue was kept on ice. Mechanically isolated myocytes were permeabilized in relaxing solution containing 0.5% Triton X-100 (5 minutes) a treatment which also removes soluble and membrane-bound kinases and phosphatases, which may alter the phosphorylation status of myofibrillar proteins. To remove Triton, cells were washed twice in relaxing solution. Subsequently, a single myocyte was attached between a force transducer and a piezoelectric motor. Isometric force measurements were performed at different [Ca^{2+}], at 15°C, and a sarcomere length, measured in relaxing solution, of 2.2 µm. Rate of force redevelopment (Ktr) was determined in activating solution (pCa values ranging from 4.5 to 5.8) when a steady level of force was developed by rapidly slackening and re-stretching the cardiomyocyte by 20% of
its length. Upon slackening force drops to zero and upon re-stretch force redevelopment occurs to the initial steady level. Force redevelopment was fitted to a single exponential to estimate $K_{tr}$.

The diameters of the cardiomyocyte were measured microscopically, in two perpendicular directions. Cross-sectional area was calculated assuming an elliptical cross-section. The composition of relaxing and activating solutions used during force measurements was calculated as described previously. The $pCa$, $-\log [Ca^{2+}]$, of the relaxing and activating solution (pH 7.1) were 9 and 4.5, respectively. Solutions with intermediate free $[Ca^{2+}]$ were obtained by mixing of the activating and relaxing solutions. After the first control activation at saturating (maximal) $[Ca^{2+}]$ ($pCa=4.5$), resting sarcomere length was readjusted to 2.2 µm, if necessary. The second control measurement was used to calculate maximal isometric tension (force divided by cross-sectional area). The next force measurements were performed at submaximal $[Ca^{2+}]$, followed by a control measurement. Force values obtained in solutions with submaximal $[Ca^{2+}]$ were normalized to the interpolated control values. Cardiomyocytes were stored on ice to prevent myofilament-bound kinases and phosphatases to alter $Ca^{2+}$-sensitivity ($pCa_{50}$) of force during the measurements.

After the first force–pCa series, part of the myocytes from Sham and MI hearts were incubated in relaxing solution containing the exogenous catalytic subunit of protein kinase A (3 µg/mL [100 U/mL]; Sigma, batch 35H9522) and 6 mmol/L dithiothreitol for 40 minutes at 20°C, after which a second force–pCa series was obtained. The mean parameters of the measurements before protein kinase A (PKA) treatment were consistent with the observations in the entire Sham and MI group studied. Force–pCa relations were fit to the Hill equation:

$$\frac{F(Ca^{2+})}{F_0} = \frac{[Ca^{2+}]^{nH}}{(C_{a50}^{nH})+[Ca^{2+}]^{nH}},$$

where $F$ is steady-state force, $F_0$ denotes the steady force at saturating $[Ca^{2+}]$, $nH$ reflects the steepness of the relationship, and $C_{a50}$ (or $pCa_{50}$) represents the midpoint of the relation.

Myosin heavy chain composition

The myosin heavy chain (MHC) isoform composition was analyzed by one-dimensional SDS-PAGE.
Myofilament Proteins phosphorylation status

To determine phosphorylation status of the myofilament proteins MyBP-C, TnT, TnI, MLC-2 and desmin, LV samples (20 µg/lane) were separated on gradient gels (4-15%) and stained with Pro-Q diamond phosphoprotein gel stain (Pro-Q; Molecular Probes) in conjunction with SYPRO Ruby staining (Molecular Probes) of the gels. To preserve the endogenous phosphorylation status, frozen tissue samples were homogenized in liquid nitrogen and re-suspended in 1 mL ice-cold (-20°C) 10% trichloroacetic acid (TCA; dissolved in acetone containing 0.1% (w/v) dithiothreitol (DTT)) solution. The phosphorylation signals for myofilament proteins were normalized to the intensities of the SYPRO Ruby stained myosin binding protein C bands. The signals were analyzed using the luminescent image analyzer las-3000 and Aida image analyzer.

β1-adrenergic Signaling

cAMP. About 5-10mg of frozen left ventricular tissue was homogenized in a liquid nitrogen cooled Teflon vial with a Teflon sphere (Mikro-Dismembrator U, setting 1700rpm, 4 minutes) in the presence of 100µl frozen 0.1M HCl. The frozen powder was collected and the vial was rinsed with the remaining 40 volumes of 0.1M HCl. The homogenate was sonicated for 30 seconds at 10 micron on ice water. An aliquot was removed for protein determination. cAMP levels were measured in 5x diluted extracts using an enzyme immunoassay kit (Assay Designs, USA) according to the manufacturer’s instructions.

PKA. About 5-20mg of frozen tissue was homogenized in the presence of 100µl frozen PKA extraction buffer (25mM Tris-HCl pH 7.4, 10mM 2-mercapto-ethanol, 1mM NaF, 100µM IBMX, 50µM Cantharidin and protease inhibitor mix Complete™ (Roche, Switzerland) similarly as described above. The homogenate in totally 20 volumes of PKA extraction buffer was sonicated for 30 seconds at 10 micron on ice water and centrifuged for 5 minutes. An aliquot of the clear supernatant was removed for protein determination. PKA levels were measured using the PepTag® Assay kit (Promega, USA) with a modified reaction buffer (1mM extra ATP), 10µg of protein and 20 minutes reaction time at room
temperature. After the reaction phosphorylated and unphosphorylated fluorescent proteins were separated on a 0.8% agarose gel and ratios of bands were determined using a Kodak Image Station 440CF equipped with Kodak 1D software. Amounts of PKA were calculated from a standard curve of pure cAMP-dependent PKA catalytic subunit.

**Western Immunoblot Analysis**

Approximately 25 mg of frozen tissue was homogenized at liquid nitrogen temperature in a microdismembrator unit (B. Braun Biotech International, 1700 rpm) for 4 min in a Teflon vial with a Teflon sphere. The frozen powder was suspended in 20 volumes of cold Laemmli loading buffer, heated for 15 min at 37° and centrifuged for 1 min at 9700·g_{av}. Aliquots of the clear supernatants were removed for protein determination using the RCDC protein assay (Bio-Rad Laboratories) and for Western blot analysis. Proteins were separated by SDS-PAGE using 10% gels, 15% gels or 7.5%-15% gradient gels. Samples were reheated for 5 min at 37°C before use and either 5µg or 20µg (depending on the antibody to be used) of protein/lane was applied onto the gels. Following electrophoresis proteins were blotted overnight at 40V onto PVDF membranes (Immunblot, Bio-Rad). Blots were stained reversibly with Ponceau Red to check protein loading and transfer. Blots were pre-incubated in TTBS (10 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 0.1% Tween-20) supplemented with 0.5% non-fat milk powder for 1 h at room temperature and incubated overnight at 4°C with diluted primary antibodies. Anti-phospholamban (PLB, mouse monoclonal) was from Affinity Bioreagents and anti-SERCA2A (rabbit polyclonal) was from Abcam. Anti-G_a_i-3 (C-10, rabbit polyclonal), anti-GRK2 (C-15, rabbit polyclonal), anti-Na^+/Ca^{2+}-exchanger 1 (NCX1, P-13, goat polyclonal) and anti-β1-adrenergic receptor (rabbit polyclonal) were from Santa Cruz Biotechnology. Phospho-specific rabbit polyclonal antibodies against a phospho-serine (P-Ser16) or phospho-threonine (P-Thr17) containing sequence of PLB were from Cyclacel. Anti-atrial natriuretic peptide was from Chemicon. A mouse atrial extract was used as positive control.

Blots were probed for 3 h at room temperature with horseradish peroxidase conjugated goat anti-rabbit or rabbit anti-goat or goat anti-mouse secondary antibody (Pierce). In between the incubations the
blots were washed extensively with TTBS. For SERCA2a, phospholamban and phospho-phospholamban detection a rat cardiac membrane preparation was used as positive control. For GRK2, \( \alpha_i-3 \) and \( \beta_1 \)-adrenergic receptor detection a rat brain extract was used as positive control. Signals were visualized using Supersignal® West Femto Maximum Sensitivity Substrate (Pierce) and Hyperfilm™ ECL (Amersham Biosciences). Signal densities were quantified using a Bio-Rad calibrated GS-800 scanner and Quantity One quantitation software (Bio-Rad).

**Contractile Properties of Intact Cardiomyocytes**

In an additional group of 6 MI\textsubscript{SED} and 5 MI\textsubscript{EX} mice, single LV cardiomyocytes were enzymatically dissociated as previously described\textsuperscript{9}; in each animal 3 to 5 cells were studied. Mice were heparinized and killed by an overdose of pentobarbital (100 mg/kg i.p.), and the hearts quickly excised. After cannulation of the aorta, hearts were mounted to a Langendorff perfusion set. The heart was briefly rinsed with normal Tyrode solution, containing (mM): 137 NaCl, 5.4 KCl, 0.5 MgCl\(_2\), 1 CaCl\(_2\), 11.8 Hepes and 10 glucose, pH 7.4. Subsequently the heart was perfused with a Ca\(^{2+}\)-free solution for 8 min. The Ca\(^{2+}\)-free Tyrode solution contained (mM): 130 NaCl, 5.4 KCl, 1.2 KH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 6 Hepes, 20 glucose, pH 7.2. Collagenase A (0.6 mg ml\(^{-1}\), Roche Diagnostics, GmbH, Mannheim, Germany) and protease (type XIV, 0.1 mg ml\(^{-1}\), Sigma, St Louis, MO, USA) were added to the Ca\(^{2+}\)-free solution and perfused for 10 min. The enzymes were washed out with low Ca\(^{2+}\) solution, i.e. the Ca\(^{2+}\)-free solution to which 0.18 mM CaCl\(_2\) was added. The hearts were removed and the non-infarcted part of the LV, including the septum was cut into small pieces and further dissociated into single cells by gentle shaking. Cells were stored in low Ca\(^{2+}\) solution at room temperature and used within 8 h after isolation. All experimental procedures were approved by the Ethics Committee on Animal Use of the University of Leuven.

Cells were placed in a perfusion chamber on the stage of an inverted microscope (Nikon Diaphot). Cell shortening was measured with a video-edge detector (Crescent, Salt Lake City, UT, USA) at 240 Hz frame rate. Field stimulation was done with 5 ms square pulses of constant voltage, at 20 % above threshold. The external solution was the normal Tyrode solution, bath temperature was 30\(^{\circ}\)C. The
absolute cell shortening is expressed as the fractional shortening, i.e. normalized to resting cell length, \( \Delta L / L_0 \).

\([\text{Ca}^{2+}]_i\) was measured in cells using whole-cell ruptured patch recording. The set-up for fluorescence recording, procedures for calibration to \([\text{Ca}^{2+}]_i\), and recording of membrane potential and currents were as described before.\(^9\) The pipette solution contained (in mmol/L): 120 K-aspartate, 20 KCl, 10 K-HEPES, 5 MgATP, 10 NaCl, and 0.05 K₅-fluo-3, pH 7.2. The external solution was the normal Tyrode solution, bath temperature was 30°C. Cells were stimulated under current clamp with 5 ms square pulses at different frequencies.
References


