Reduced Force Generating Capacity in Myocytes From Chronically Ischemic, Hibernating Myocardium

Virginie Bito,* Jolanda van der Velden,* Piet Claus, Christoph Dommk, Alfons Van Lommen, Luc Mortelmans, Erik Verbeken, Bart Bijnens, Ger Stienen, Karin R. Sipido

Abstract—The contractile dysfunction of the hibernating myocardium in situ results from local environmental factors, but also from intrinsic cellular remodelling that may determine reversibility. Previous studies have suggested defects in myofilament Ca\textsuperscript{2+} responsiveness. We prepared single myocytes from control (CTRL, \(n_{\text{pigs}}=7\)) and from hibernating myocardium (HIB, \(n_{\text{pigs}}=8\)), removed the membranes and measured isometric force development during direct activation of the myofilaments. One- and 2-dimensional polyacrylamide gel electrophoresis and specific phosphoprotein immunoblotting were performed on tissue homogenates from matched samples. Cellular ultrastructure was evaluated using electron microscopy. Normalized for cross-sectional area, passive force was not different but maximal isometric force was significantly reduced in myocytes from HIB (11.6±1.5 kN/m\textsuperscript{2} versus 18.7±1.6 kN/m\textsuperscript{2} in CTRL, \(P<0.05\)). Ca\textsuperscript{2+} sensitivity and steepness of the normalized force-pCa relationship were not different, and neither was the rate of force redevelopment (\(K_r\)). No alterations were observed in isoform expression, phosphorylation or degradation of specific myofilibrillar proteins. However, in HIB samples the total protein volume density was decreased by 23% (\(P<0.05\)). Histology showed glycogen accumulation and electron microscopy confirmed a reduction in myofilament density from 69.9±1.9% in CTRL to 57.1±0.9% of cell volume in HIB (\(P<0.05\)). In conclusion, decreased potential for force development in the hibernating myocardium is related to a reduction of myofilibrillar protein per cell volume unit with replacement by glycogen and mitochondria. These changes may contribute to slow functional recovery on revascularization.

Key Words: hibernation ■ contractile function ■ myocytes ■ calcium

In the heart with coronary artery disease (CAD), hibernating myocardium has been defined as those areas of the heart distal to a severe coronary stenosis that have reduced contractile function but no significant necrosis and that will recover function after revascularization.\(^1\) The presence of hibernating myocardium can be demonstrated by nuclear imaging showing preserved metabolic activity and sometimes even enhanced glucose uptake, together with reduced perfusion, if not at rest, then markedly so during a challenge.\(^3\) Another hallmark of hibernating myocardium that helps identifying areas that would benefit from revascularization, is stress echocardiography. During dobutamine infusion, contraction of the hibernating myocardium can increase at low doses, but it will decrease at higher doses, because of the reduced flow reserve in the area,\(^5\) distinguishing hibernating from necrotic or stunned myocardium. When a strict distinction is applied between regions that have a reduced perfusion at rest, defined as true hibernation, and regions with normal perfusion at rest (chronic stunning), an initial positive inotropic response to low dose dobutamine can be seen in the latter but is mostly absent in the former (reviewed in\(^6\)).

The prevalence of hibernating myocardium in the population with CAD is high, and it is a challenge for treatment.\(^7,8\) Indeed, without revascularization, mortality in CAD patients with hibernating myocardium is higher than in CAD patients without viable myocardium.\(^9\) The mechanisms underlying the reduced contractile function and increased mortality remain incompletely understood. Both changes in the local environment in vivo and intrinsic remodelling of the myocytes are likely to contribute.

Studies on human biopsies of hibernating myocardium from patients undergoing bypass surgery have focused on the structural changes and altered gene regulation. Hibernating myocardium contains a variable number of cells with altered ultrastructure, with loss of myofilibrillar proteins, swollen mitochondria and glycogen accumulation.\(^10-12\) These changes have been interpreted as cell de-differentiation with altered gene expression and reexpression of fetal structural genes\(^10,13\)
but also as signs of degeneration.\textsuperscript{11} Apoptosis and compensatory hypertrophy are also observed, although there is some controversy about the extent and significance of apoptosis.\textsuperscript{10,13–15} There are no functional studies in vitro on human tissue or cells from hibernating human myocardium.

Despite the difficulties involved in exactly reproducing hibernation in humans, a number of valid animal models have been developed (reviewed in\textsuperscript{16}). Canty, Fallavollita and co-workers have characterized a pig model in which coronary stenosis develops slowly during the animal growth over several months.\textsuperscript{17,18} SERCA, phospholamban and RyR2 mRNA and protein expression were reduced, but troponin I (TnI) was not degraded.\textsuperscript{19,20} These authors also observed apoptosis and compensatory cellular hypertrophy.\textsuperscript{21} The functional characteristics of the hibernating myocardium in those models have been studied in vivo, but there are no in vitro data on isolated tissues or cells that characterize the functional remodeling.

We recently described a closed-chest model of hibernation in the pig where we isolated myocytes to study function in vitro.\textsuperscript{22} We could thus demonstrate that the decreased function in vivo is at least partly related to intrinsic remodeling of the myocytes. Contraction of the myocytes was blunted, the L-type Ca\textsuperscript{2+} current, \(I_{\text{Ca,L}}\), and Ca\textsuperscript{2+} release from the sarcoplasmic reticulum were reduced. Yet contraction amplitude of the intact cells could not be restored by increasing available Ca\textsuperscript{2+}, suggesting there was additional impairment of myofilament function. We therefore in the present study use myocyte preparations devoid of membranes to characterize directly the myofilament function. We observe a reduced maximal force development that is not related to protein isoform shift or phosphorylation but results at least to a large extent from a reduction of myofilament density and ultrastructural reorganization.

**Materials and Methods**

**Animal Model**

The animal model is described in detail on the online data supplement available at http://circres.ahajournals.org. In brief, a copper-coated stent inducing intima proliferation and stenosis was inserted into the circumflex artery of young pigs (body weight 20 to 25 kg). The posterior wall developed the hallmarks of hibernating myocardium. At the time of euthanasia after 4 to 6 weeks (body weight 36 to 67 kg), several tissue samples (\(\pm 5 \text{ mm} \)) were taken from the hibernating area (HIB, \(n_{\text{animals}}=8\)) and from the same area in weight-matched control animals (CTRL, \(n=7\)). The samples were frozen immediately at \(-80^\circ\text{C}\). From the remaining tissue single intact cells were enzymatically isolated for studies on Ca\textsuperscript{2+} handling as reported before.\textsuperscript{22} In 2 HIB and 3 CTRL animals, additional samples were processed for histology.

**Myocyte Isolation and Skinning**

Single skinned myocytes were prepared from the frozen tissue samples as described previously\textsuperscript{23} and detailed in the online data supplement available at http://circres.ahajournals.org; half of the sample was used for myocyte preparation and half of the sample for protein studies.

**Measurement of Isometric Force**

In skinned myocytes, the myofilaments are directly activated by the [Ca\textsuperscript{2+}] of the bathing solution. The experiments were performed as described previously,\textsuperscript{23} and detailed in the online supplement.

![Figure 1. Measuring force in skinned myocytes. A, Example of a skinned myocyte attached between a piezoelectric motor and a force transducer. B, Representative recordings obtained from a CTRL and HIB skinned myocyte during superfusion with pCa 4.5 or pCa 5.4. When immersed in the activating solution, force developed to reach a steady state. C, Sudden drop of force when cell length was rapidly reduced by 20% and the subsequent force redevelopment was fitted with an exponential curve from which we determined \(K_r\). The sudden changes in force represent the transition through the interface between the solutions and the air.](http://circres.ahajournals.org/)

Briefly, isometric force was measured in the skinned myocytes attached between a piezoelectric motor and a force transducer (Figure 1A, B) at 15°C at a sarcomere length set at 2.2 \(\mu\text{m}\). The isometric force developed was normalized to the cross-sectional area of the myocyte. Force–pCa relations were fit with a modified Hill equation:

\[
\frac{F_{\text{max}}}{F_0} = \frac{[\text{Ca}^{2+}]^{m}}{[\text{Ca}^{2+}]^{m}+[\text{Ca}^{2+}]^{n}}
\]

Rate of force redevelopment (\(K_r\)) was measured during steady state activation, at different pCa; sampling rate was at 1 kHz. After full force development, the myocyte length was suddenly reduced by 20% for 3 ms. The restretching of the preparation to its original length induces force redevelopment to the maximal force (Figure 1C). The passive force was measured in relaxing solution.

**Protein Expression and Phosphorylation**

Tissue homogenates were prepared from freeze-dried tissue, obtained from the same samples used for myocyte isolation, and diluted in sample buffer to a concentration of 1 \(\mu\text{g/\muL}\) (dry weight/volume). Protein concentration was determined by using a Bio-Rad DC protein assay, calibrated with bovine serum albumin.\textsuperscript{24}

The procedure for 2-dimensional polyacrylamide gel electrophoresis was as used in\textsuperscript{25} and is detailed in the online data.
supplement. The protocols for specific Western blots and for measuring the degree of phosphorylation can likewise be found in the online supplement.

In a previous study, the phosphorylation status in a tissue homogenate was compared with that in mechanically isolated Triton-treated cardiomyocytes prepared from the same tissue. The 2D-gels did not reveal differences in the phosphorylation status of myofilament proteins between the 2 sample types.

**Histological Data**

For light microscopy, the samples were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 4 μm thickness. For electron microscopy, tissue samples were minced into 1 mm³ pieces and fixed in 2.5% glutaraldehyde. The further procedures are detailed in the online data supplement available at http://circres.ahajournals.org.

The methods for determining volume density of myofilaments and mitochondria and for glycogen quantification are detailed in the online supplement.

**Statistical Data**

All the data are shown as mean±SEM. According to the type of experiments, we used either paired, unpaired t-test or 2-way ANOVA followed by post-hoc Bonferroni test. The data were considered significant for P<0.05.

**Results**

Are the Intrinsic Properties of the Myofilaments Altered With Chronic Ischemia?

Passive force, active force development and rate of force redevelopment were measured in 31 cells from 8 HIB and in 27 cells from 7 CTRL; data are summarized in Figure 2. The passive force was not different (Figure 2A). The maximal Ca²⁺-activated force was significantly reduced in HIB (18.7±1.6 kN/m² in CTRL versus 11.6±1.5 kN/m² in HIB, P<0.05; Figure 2B). Figure 2C shows the normalized developed force as a function of [Ca²⁺]; Ca²⁺ sensitivity was not different between CTRL and HIB (pCa₅₀=5.49±0.02 in CTRL versus 5.47±0.03 in HIB) and the steepness of the relation was comparable (n₅₀=3.21±0.23 in CTRL versus 3.12±0.43 in HIB).

The rate of force redevelopment (Kᵣ) was determined at pCa between 4.5 and 5.6. Kᵣ decreases with a decrease in [Ca²⁺], but this relation was comparable between CTRL and HIB, suggesting that the cross-bridges kinetics are not altered in HIB (Figure 2D). The relation between Kᵣ and active force was linear in CTRL and HIB, approximately parallel, showing a leftward shift in HIB (not shown) as expected from a proportional decline in force generating capacity of the cells.

The isolation procedure of the current study was different from our previous study where the myocytes were enzymatically isolated. To test for a selection bias and to check we were studying a similar population of myocytes, we also performed the skinning procedure on myocytes obtained by enzymatic isolation. We compared tension-pCa curves of myocytes obtained with the 2 different procedures and found no differences (see online supplement). Therefore, the results we obtained from skinned preparations can be related to the previous data we obtained in enzymatically isolated cells.

Is the Decrease in Maximal Force Development Because of a Change in Phosphorylation Status of the Myofibrillar Proteins?

We looked for potential changes in the phosphorylation status of myosin light chain 1 and 2 (MLC-1 and MLC-2) and troponin T (TnT), by analyzing 2-dimensional gel electrophoresis of homogenates. These were prepared from the same biopsies that were used for preparation of skinned myocytes; each frozen sample was divided in 2 parts, 1 for myocyte preparation and the other for protein studies. Figure 3A is an example of a 2D-gel from a CTRL sample. The data are summarized in Figure 3B. We found no evidence for changes in MLC-1, MLC-2 and TnT phosphorylation in HIB. We excluded degradation in the samples by measuring the ratio of MLC-1/MLC-2 and TnT/actin, which were unchanged (Figure 3C).

We further investigated TnI phosphorylation by using specific antibodies against total TnI and dephosphorylated TnI. The results are expressed as the ratio of dephosphorylated TnI to total TnI. As summarized Figure 4A, we found no difference between CTRL and HIB (ratio 22B11/16A11=0.44±0.10 in 4 CTRL versus 0.46±0.14 in 4 HIB). With the Pro-Q Diamond staining method (see online supplement) we evaluated the degree of phosphorylation of myosin binding protein C (MyBP-C), reported to be altered during ischemia/reperfusion. The values for phosphorylated MyBP-C did not differ between CTRL and HIB (n=5; 0.076±0.004 and HIB (n=5; 0.071±0.007) samples (Figure 4B). With the same approach we also could not detect differences in total TnI phosphorylation (HIB 0.04±0.01 versus CTRL 0.06±0.02, n=5 each). In separate samples we checked total titin phosphorylation and saw no difference (HIB, n=2; 0.33±0.01 versus CTRL, n=2; 0.32±0.01).
We also looked for TnI degradation by immunoblot and quantification of the 25 kDa band of degraded TnI, as described before. In the present samples we again could not detect a significant increase in TnI degradation (data not shown).

To evaluate the cytoskeletal component we measured expression of desmin, α-actinin in relation to actin. We found no difference between HIB and CTRL (Figure 4C). In separate gels we also evaluated titin isoform expression and found that the ratio of NB2A:NB2 was close to 1 and not different between groups (data in online supplement).

For a potential shift in isoform of myosin heavy chain we analyzed 1D silver-stained protein gels. In both groups we observed only one band, corresponding to the β-isofrom of the myosin heavy-chain. From the same gels, the TnC/actin ratios were measured and found similar for CTRL and HIB (Figure 4D).

Is There a Global Reduction of Myofibrillar Protein Density?
For the 2D-gel electrophoresis and immunoblots, equal amounts of protein were loaded onto the gels. This approach would not allow detection of a global reduction of myofibrillar protein density in the myocytes. As myofibrillar proteins make up the majority of total myocyte protein, normalization for total protein concentration of the tissue samples could obscure potential differences. We therefore measured total protein content per volume, as an indicator for the density of myofibrillar protein in the tissue (Figure 5A). Total protein concentration was significantly reduced in HIB compared with CTRL \( (P<0.05) \). In another approach we examined the relation between total protein concentration in homogenates and the force developed by the myocytes isolated from the same hearts. This relation showed that the low force developed in HIB corresponded with low protein content (Figure 5B).

Ultrastructural Changes
The data above suggest that there might be a global reduction of myofibrillar protein density, and we therefore examined the tissue for ultrastructural changes. In the light microscopy sections with PAS staining, we saw in tissues from HIB a large fraction of cells that had clear accumulations of glycogen, whereas this was seen to a much lesser extent in CTRL (Figure 6A).

Studies of thin sections with electron microscopy confirmed the glycogen accumulation and showed in addition alterations in mitochondrial structure (Figure 7A). Quantifi-
cation revealed a decrease in myofilament density in HIB (57.1 ± 0.9% of surface area in HIB versus 69.9 ± 1.9% in CTRL, P<0.05) and an increase of the area occupied by mitochondria (34.4 ± 0.5% of surface area in HIB versus 29.7 ± 1.2% in CTRL, P<0.05) (Figure 7B). The above data are consistent with the hypothesis that the amount of myofilaments per unit of volume is decreased. We also quantified glycogen accumulation using a semiquantitative scoring (see online supplement) confirming the higher glycogen content in HIB.

In our previous analysis of enzymatically isolated single cells, we noticed that cell length and width were significantly increased.22 We also measured cell width in the PAS stained sections and it was significantly larger in HIB (25.4 ± 0.7 μm versus 22.1 ± 0.7 μm in CTRL, P<0.05, Figure 6B). In the isolated skinned myocytes, we also found an increase in primary cell dimensions (Figure 8A), statistically significant for length, width, thickness (P<0.05), and also for the calculated cross-sectional area (Figure 8B).

**Discussion**

**Reduced Force Development and Underlying Mechanisms**

We previously reported on the reduced contraction of intact single myocytes isolated from the hibernating myocardium in the pig and postulated that in addition to altered Ca\(^{2+}\) handling, an intrinsic defect in myofilament function could be present.22 In the current study we establish that there is indeed a reduced maximal response to Ca\(^{2+}\) activation without change in the sensitivity to Ca\(^{2+}\).
The latter was a somewhat surprising finding as we had expected alterations in sensitivity, potentially result from changes in phosphorylation of TnI, as previously described. The 2D-gel electrophoresis and specific immunoblots for TnI phosphorylation however did not reveal changes in phosphorylation, consistent with the functional data. There was no evident shift in isoform expression either and changes in MyBP-C were also not observed.

A potential explanation for the reduction of maximal force could be degradation of TnI. As we had previously reported, we could not detect an increase in TnI degradation, but it must be stressed that the immunoblot approach may not be sensitive enough to pick up a modest increase that may nevertheless be of functional relevance. The recent development of assays to directly quantify oxidation of myofibrillar protein also warrants further study of the potential role of oxidative modifications.

Because passive and restoring forces are mainly due to titin, our findings of unaltered passive force and rate of force redevelopment in HIB, suggested that titin function was preserved. Protein analysis confirmed the absence of an isoform switch or changes in phosphorylation. This is in line with recent studies linking isoform switches in titin to changes in passive force in cardiomyofibrils and single skinned cardiomyocytes, and studies showing that PKA-mediated phosphorylation of titin alters passive force. Our current data suggest that the reduced force development is mainly the result of a decrease in myofilament volume density. The loss of force development per cross-sectional area in individual myocytes is around 35%. With the current techniques it is difficult to quantify the reduction in myofibrillar protein also warrants further study of the potential role of oxidative modifications.

A recurrent question and discussion point with regard to the use of isolated cells is whether the myocytes are representative for the entire myocardium. It is indeed a limitation.
of the technique that only a small number of myocytes can be studied per sample, and there is the possibility that cells from the hibernating myocardium with more extensive changes might be less resistant to the isolation procedure. We looked at the relative yield of myocytes during isolation, considering that a selective loss of the more damaged cells would lower the ratio of viable to total number of myocytes. As we could not observe differences between HIB and CTRL, we assume that our isolation is not biased in this way. We cannot examine glycogen loading in the same cells as used for functional studies, but in two HIB hearts we stained a sample of isolated cells and could see abundant glycogen in a number of cells (data not shown), suggesting that the isolation is not skewed toward less affected myocytes.

The histology data are consistent with previous data in humans, and those data predicted a loss of contractile function. However, our data are the first to actually measure force development in isolated myocytes.

The Role of Hypertrophy and Reduced Myofilament Density

We previously reported that the myocytes isolated from HIB were hypertrophied, in line with histological studies on tissue from human hearts and from animal models. The cells used in the current study again were larger than their controls. The increase in cell diameter may actually partially compensate for the reduction in myofilament density, as the amount of force per cell is not significantly smaller in HIB. It is conceivable that the synthesis of contractile proteins is deregulated in relation to the total increase in cell size.

Glycogen loading and alterations in mitochondria occupying a larger fraction of the cytosol may also lead to reduced myofilament density. These alterations are likely to result from activation of a complex signaling, including activation of the hypertrophic response through increased loading by the surrounding myocardium. A recent study linked activation of the p38-MAPK axis to increased glucose uptake, a factor contributing to glycogen loading. Glycogen synthesis is further promoted by a reduction in the GSK-3β activity. As the flow reserve is significantly reduced in hibernating myocardium, intermittent episodes of acute ischemia may in addition provide a unique stimulus for remodelling, aimed at enhanced survival.

Relation to Previous Findings

Our observation of a reduction in maximal force is in line with the data of Heusch and colleagues who measured a reduction in Ca²⁺ responsiveness of contraction in situ in their short-term model for hibernation. Such changes were not investigated in the chronic hibernation model, but there alterations in Ca²⁺ handling, namely a reduction in SERCA and RyR were seen. In our model we reported a reduction in Ca²⁺ current density. This altered Ca²⁺ handling will further exacerbate the myofilament defect. Furthermore, subcellular heterogeneity in Ca²⁺ handling may be present and potential alterations in T-tubule density could contribute to enhanced dyssynchrony.

Relation to In Vivo Function

In vivo, the rate of pressure development in the HIB animals was significantly reduced. Analysis of the local wall deformation by strain rate indicated that the rate of wall thickening was reduced. Our measurements of the rate of force redevelopment on relengthening could not detect any differences between HIB and CTRL, indicating that the intrinsic crossbridge kinetics were not different. In intact cells however, the rate of shortening was reduced. This suggests that the slowing of contraction is related to the changes in excitation-contraction coupling. In vivo, enhanced loading imposed by the surrounding normal myocardium is also an important factor which may further exacerbate the extent of contractile dysfunction of the hibernating myocardium.

Limitations of the Study

In our study we examined the region of interest only and compared with the equivalent region in normal controls. With this approach we avoided confounding factors such as regional differences in function and the presence of remodelling in the remote myocardium. Indeed, whereas the observed differences can explain changes in local contractile function, the overall changes in contractile function of the left ventricle may also be determined by changes in the remote myocardium.

Conclusions and Perspectives

Decreased potential for force development in the hibernating myocardium is related to a reduction of myofibrillar protein...
per cell volume unit, associated with structural reorganization. Previous studies have linked the potential for recovery of contractile function after revascularization to the extent of structural changes in biopsies taken at the time of surgery, in particular fibrosis, and to a lesser extent the number of de-differentiated cells. It has remained unclear whether the de-differentiated cells were actually viable cells with the potential for recovery.

Although this and our previous study have not addressed recovery, our data indicate that the de-differentiated cells retain the basic elements of excitation-contraction coupling, even if the ultrastructural changes are profound. This suggests that these myocytes have the potential for recovery, but also that recovery may be slow.

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Disclosures
No.

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EXPANDED METHODS

Animal Model

Our animal model is based on the approach used by Weidemann et al. 1, where a copper-coated stent is implanted in the circumflex artery of young pigs (body weight 20-25 kg) inducing a severe coronary stenosis within 7-10 days. The protocol for copper-coating was slightly modified in that electroplating was of shorter duration and intensity (1 min at 100 mA, vs. 1.5 min and 150 mA) and that stents were heparinized (dipped in heparin solution 5000 IU/mL, followed by air-drying, repeated 3 times), resulting on average in a lower degree of stenosis and a low incidence of myocardial infarction compared to2.

Animals received a loading dose of 375 mg clopidogrel and 600 mg acetylsalicylic acid one day before the intervention and during the follow up a daily dose of 75 mg clopidogrel and 300 mg acetylsalicylic acid was given until the day of sacrifice.

We identified animals with hibernation (HIB) on the basis of a moderate decrease in baseline contractility, the stress response and additional PET and histology data. The mean degree of stenosis in the HIB group (n=8) described in the present paper was 90±2 %; we didn’t find collateral formation in any of the HIB animals. The overall in vivo functional data are shown in Table I. The baseline strain in the posterior wall was 44±2 % and significantly reduced compared to control animals (60±5%); note that this value is significantly higher than in the previously described group with non-transmural infarction (32±6%)3. The stress response of HIB animals is illustrated in Supplemental Figure I for five animals. The dobutamine response is flat in the lower dose range, with a sharp decrease at 20 μg/kg dobutamine (ANOVA for the curves, P<0.01). The dobutamine
stress response of the other animals used in the present study was evaluated with a
different imaging protocol precluding pooling of the data, but had similar characteristics.
Of the five animals represented in Figure 1, two were used for PET study of perfusion and complete pathology studies together with two other animals not included in the
class. The perfusion study was performed using a whole-body PET camera (model 931-08/12, CTI Siemens, Knoxville, USA). Fifteen mCi of $^{13}$NH$_3$ in 5 ml saline was slowly
infused at a constant rate of 10 ml/min followed by a 20-ml flush of saline at the same
rate. Acquisition was started simultaneously with the injection of $^{13}$NH$_3$. Total acquisition
time was 30 minutes. From the delineated images, polar maps were derived, representing
the $^{13}$NH$_3$ uptake in a single two-dimensional image. The reference region of normal
tracer uptake was defined on the ammonia polar map as the region with maximal uptake
in the remote area and the mean value of that region was set as 100%; results are thus
given as relative flow in %. In these four animals, the flow as measured with $^{13}$NH$_3$ in the
area perfused by the stenosed coronary artery was $82\pm4$ % compared to $90\pm3$ % in the
remote area ($P<0.02$; Supplemental Figure II). The phenotype of these animals is thus
consistent with hibernation in the stricter sense as reviewed in. On histological
examination of transmural tissue slices throughout the posterior wall of these animals,
there was no evidence of myocardial infarction but for some minor foci of necrosis
subendocardially; there was increased glycogen staining in the subendocardial and
midmyocardial layers. From the animals that were used for the cellular studies we
examined transmural biopsies confirming the findings described above; the glycogen
loading was further quantified in electron microscopy described in the present study.
At the time of sacrifice after 4-6 weeks (body weight 36-67 kg), several tissue samples (±5 mm$^3$) were taken from the hibernating area (HIB, n$^{\text{animals}}$=8) and from the same area in weight-matched control animals (CTRL, n=7). The samples were frozen immediately at -80ºC. From the remaining tissue single intact cells were enzymatically isolated for studies on Ca$^{2+}$ handling; these results are reported in 6. In 2 HIB and 3 CTRL animals, additional samples were taken for histology and stored in the appropriate fixation solution (see below).

*Myocyte isolation and skinning*

Single skinned myocytes were prepared from the frozen tissue samples; during the whole procedure, the tissue was kept on ice. The tissue was first thawed in a Ca-free relaxing solution (in mmol/L: free Mg$^{2+}$ 1, KCl 145, EGTA 2, ATP 4, imidazole 10, pH=7) and myocytes were mechanically isolated by pottering the tissue sample. Isolated myocytes were then immersed for 5 min in the same solution with 0.5% Triton X-100 added to permeabilize and remove lipid membranes, followed by careful washout.

*Measurement of isometric force*

Isometric force was measured in the skinned myocytes attached between a piezoelectric motor and a force transducer at 15°C at a sarcomere length set at 2.2 µm; sampling rate for steady state data was 20 Hz. At this temperature preparations are stable and sarcomere behaviour uniform$^7$; the sarcomere length of 2.2 µm is close to optimal length$^8$. Width and depth of the cardiomyocyte were measured microscopically; cross-sectional area of the cardiomyocytes was calculated assuming an elliptical cross-section. The
composition of the relaxing and the activating solution was calculated as described previously\textsuperscript{9}; pCa of the relaxing and maximally activating solution was respectively 9 and 4.5. Solutions with intermediate [Ca\textsuperscript{2+}] were made by mixing the relaxing solution with the activating solution in appropriate proportions.

The experiments were performed as described previously\textsuperscript{10}. Briefly, the sarcomere length was checked after a first activation by a pCa 4.5 solution and readjusted to 2.2 µm if necessary. The next control measurement at pCa 4.5 was used to measure maximal isometric force developed. This was followed by 6 measurements at different pCa applied randomly. The experiment was ended by re-application of pCa 4.5. The difference between the first and the last pCa 4.5 was used to measure the run-down of the response.

The isometric force developed was normalized to the cross-sectional area of the myocyte, and corrected for the run-down. Cells with a run-down >20 % were not used. Force–pCa relations were fit with a modified Hill equation:

\[
\frac{F}{F_0} = \frac{[Ca^{2+}]^{nH}}{(Ca_{50}^{nH}+[Ca^{2+}]^{nH})}
\]

where F is the force at steady-state, F\textsubscript{0} denotes the steady-state force at saturating Ca\textsuperscript{2+}, nH reflects the steepness of the relationship, and Ca\textsubscript{50} represents the midpoint of the curve.

Rate of force redevelopment was measured during steady state activation, at different pCa; sampling rate was 1 kHz. After full force development, the myocyte length was suddenly reduced by 20% for 3 ms, as illustrated in Fig. 1B, right panel. This slackening induces cross-bridges uncoupling and therefore, the force drops to zero. The re-stretching of the preparation to its original length induces force redevelopment to the maximal force. K\textsubscript{tr} is derived from the fitting of force redevelopment. The passive force was
measured in relaxing solution by slackening the myocyte for 10 s and re-stretching it to its original length.

In a subset of experiments from CTRL samples, we examined the effect of the isolating procedure on the force production. Cells isolated enzymatically, as described in our previous work⁶ were skinned and used for force measurements. The data were compared to myocytes mechanically isolated and skinned, as described in the present study. These results are presented in Table II of the Online Supplement.

**Protein expression and phosphorylation**

Tissue homogenates were prepared from freeze-dried tissue, obtained from the same samples from which myocytes were isolated, and diluted in sample buffer to a concentration of 1 µg/µl (dry weight/volume). Protein concentration was determined by using a Bio-Rad RC DC protein assay, calibrated with bovine serum albumin¹¹. For two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), the samples were first treated with tri-chloro-acetic acid (TCA), to fixate the phosphorylating status of the proteins, and homogenised. The samples were loaded on immobiline strips with a pH gradient from 4.5 to 5.5 (Amersham Pharmacia Biotech, Uppsala, Sweden), in order to separate the proteins by isoelectric focussing. The second dimension of the gels was the separation of the proteins by their molecular weight, using SDS-PAGE¹². Gels were stained with Coomassie blue, scanned and analyzed using Image Quant (Molecular Dynamics).
Reduced contractile force of hibernating myocytes

Total troponin I, dephosphorylated troponin I and degraded troponin I were identified and semi-quantified by immunoblot (primary antibodies respectively clone 16A11, 22B11, both from Research Diagnostics Inc., and 8I-1, from Spectral Diagnostics Inc.).

To reveal proteolysis of desmin, α-actinin and actin similar amounts of protein (determined by the RCDC protein assay, Bio-Rad Laboratories) were loaded on 15% one-dimensional mini gels. Proteins were separated and transferred to Hybond-ECL nitrocellulose membranes. Blots were incubated with primary mouse monoclonal antibodies against desmin (5 µg protein/lane; clone DE-U-10, diluted 1:200, Sigma), actin (10 µg protein/lane; clone KJ43A, diluted 1:200, Sigma) and α-actinin (10 µg protein/lane; clone EA-53, diluted 1:200, Sigma) and signals were visualized using a secondary horseradish peroxidase-labeled goat-anti-mouse antibody and enhanced chemiluminescence (ECL plus Western blotting detection, Amersham Biosciences).

To determine phosphorylation status of myosin binding protein C (MyBP-C), samples (10 and 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. The phosphorylation signals for MyBP-C were normalized to the intensities of the SYPRO® Ruby stained MyBP-C bands. This method was also used to determine potential changes in titin phosphorylation.

The signals on the Western blots and gradient gels were analyzed using the luminescent image analyzer las-3000 and Aida image analyzer.

For evaluation of titin, tissue samples were homogenized in 50 to 100 µL of Tris-SDS buffer (pH 6.8) containing 8 µg/mL leupeptin (Peptide-Institute, Osaka, Japan). Titin isoforms were separated on agarose-strengthened 2% SDS-polyacrylamide gels and
stained with Coomassie brilliant blue. Gels were digitized, and the optical volume of protein bands was determined with Total Laboratory software (Phoretix, Nonlinear Dynamics, Newcastle upon Tyne, UK).

**Histological data**

For light microscopy, the samples were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 4 µm thickness. Successive sections were stained alternatively with hematoxylin-eosin, periodic acid-Schiff’s reagent (PAS) for glycogen and PAS after treatment with α-amylase. Cell width was measured as the diameter perpendicular to the long axis of the myocyte section.

For electron microscopy, tissue samples were minced into 1mm³ pieces and fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide and embedded in epoxy-resin. One µm sections were stained with toluidine blue and adequately prepared myocardial tissue randomly selected by light microscopy. From these, ultra thin sections of 65-70 nm were prepared, stained with uranyl acetate and lead citrate and examined in the electron microscope. From at least 3 samples per animal, random photographs (25 µm by 21 µm) were taken at 4400x magnification. The volume density of myofilaments and mitochondria was determined by a point counting method at the intersections of an orthogonal grid with vertical and horizontal lines (every 5 and 2.5 µm for myofilaments and mitochondria respectively). The % of points hitting the structures directly yields then the surface and volume density. Blood vessels were excluded from these measurements. We performed a semi-quantitative analysis of glycogen deposits in the electron micrographs of 2 HIB and 3 CTRL hearts. From every heart we randomly took 6 sample
micrographs. On each micrograph we superimposed an orthogonal grid with 270 squares of 1 \( \mu \text{m}^2 \). Within this grid 63 squares, evenly divided across the grid, were scored for the presence of glycogen: 0 = no glycogen; 1 = dispersed glycogen dots; 2 = aggregates covering more than 20% of the 1 \( \mu \text{m}^2 \) squares. This resulted in 378 squares or areas scored per heart. The person scoring was blinded towards the origin of the samples.

Statistical data

All the data are shown as mean±SEM. According to the type of experiments, we used either paired, an unpaired t-test or a 2-way ANOVA followed by post-hoc Bonferroni test. The data were considered significant for P<0.05.
Reference List


10. van der Velden J, Klein LJ, van der Bijl M, Huybregts MA, Stooker W, Witkop J, 
Eijsman L, Visser CA, Visser FC, Stienen GJ. Force production in mechanically 
isolated cardiac myocytes from human ventricular muscle tissue. *Cardiovasc Res.* 

11. van der Velden J, Merkus D, Klarenbeek BR, James AT, Boontje NM, Dekkers 
DH, Stienen GJ, Lamers JM, Duncker DJ. Alterations in myofilament function 
contribute to left ventricular dysfunction in pigs early after myocardial infarction. 

12. van der Velden J, Klein LJ, van der Bijl M, Huybregts MA, Stooker W, Witkop J, 
Eijsman L, Visser CA, Visser FC, Stienen GJ. Isometric tension development and 
its calcium sensitivity in skinned myocyte-sized preparations from different regions 
Supplemental data

Online Table I. In vivo characteristics

<table>
<thead>
<tr>
<th></th>
<th>LVEDD (mm)</th>
<th>Posterior WT (mm)</th>
<th>Septum WT (mm)</th>
<th>Systolic strain (%)</th>
<th>+dP/dt (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTRL</td>
<td>42±1</td>
<td>3.9±0.1</td>
<td>6.6±0.2</td>
<td>60±5</td>
<td>1912±70</td>
</tr>
<tr>
<td>HIB</td>
<td>39±1</td>
<td>5.6±0.6</td>
<td>7.1±0.4</td>
<td>44±2*</td>
<td>1637±73*</td>
</tr>
</tbody>
</table>

LVEDD: Left ventricular end-diastolic diameter
Posterior WT: Posterior wall thickness
Septum WT: Septum wall thickness
n=7 animals for HIB, n=7 for CTRL (for one animal in each group we have no pressure data available; we show matched data for all parameters)
* P<0.01

Online Table II. Comparison of force development in myocytes obtained with 2 different isolation methods

<table>
<thead>
<tr>
<th></th>
<th>pCa\textsubscript{50}</th>
<th>n\textsubscript{Hill}</th>
<th>Maximal Force (kN/m\textsuperscript{2})</th>
<th>Passive Force (kN/m\textsuperscript{2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic isolation</td>
<td>5.51±0.03</td>
<td>2.01±0.56</td>
<td>21.7±3.5</td>
<td>3.3±0.6</td>
</tr>
<tr>
<td>Mechanical isolation</td>
<td>5.53±0.02</td>
<td>2.64±0.19</td>
<td>16.0±1.41</td>
<td>3.3±0.6</td>
</tr>
</tbody>
</table>

n\textsubscript{cells}=8 for enzymatic isolation followed by skinning
n\textsubscript{cells}=19 for mechanical isolation followed by skinning

Online Table III. Quantification of glycogen

<table>
<thead>
<tr>
<th></th>
<th>Score 0</th>
<th>Score 1</th>
<th>Score 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTRL</td>
<td>62.4±6.5%</td>
<td>30.8±4.2%</td>
<td>6.8±3.2</td>
</tr>
<tr>
<td>HIB</td>
<td>27.3±4.8% *</td>
<td>52.3±1.6% *</td>
<td>20.1±6.4% (P=0.07)</td>
</tr>
</tbody>
</table>

Score 0: no glycogen
Score 1: dispersed glycogen dots
Score 2: aggregates of glycogen occupying more than 20% of the area
n\textsubscript{CTRL}=3 animals and n\textsubscript{HIB}=2 animals
* P<0.05
Online Figure I: Strain response to a dobutamine challenge for control animal (CTRL; n=5) and hibernation (HIB; n=5) in the segments at risk; mean ± SEM. ANOVA for Repeated Measures shows a significant difference between the two groups for the dobutamine response curves (P=0.0024). * denotes P<0.05 within the groups versus baseline; post-hoc test (Newman-Keuls).
**Online Figure II:** Myocardial perfusion of segments at risk compared to remote segments. The reference value of 100% is the maximal value observed the remote area. * P<0.02.
Online Figure III: Upper panel. Example of the separation of the two titin isoforms in a hibernation (HIB) and in a control (CTRL) sample. Lower panel. Pooled data expressed as the % of N2BA isoform of total titin in hibernation (HIB; n=5) and in control animals (CTRL; n=5); mean ± SEM.