Increased contractility and calcium sensitivity in cardiac myocytes isolated from endurance trained rats

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Abstract

Objective: Regular exercise enhances cardiac function and modulates myocyte growth in healthy individuals. The purpose of the present study was to assess contractile function and expression of selected genes associated with intracellular Ca²⁺ regulation after intensity controlled aerobic endurance training in the rat. Methods: Female Sprague–Dawley rats were randomly assigned to sedentary control (SED) or treadmill running (TR) 2 h per day, 5 days per week for 2, 4 or 13 weeks. Rats ran 8-min intervals at 85–90% of VO₂max separated by 2 min at 50–60%. Myocyte length, intracellular Ca²⁺ (Fura-2), and intracellular pH (BCECF) were measured in dissociated cells in response to electrical stimulation at a range of stimulation rates. Results: The increase in VO₂max plateaued after 6–8 weeks, 60% above SED. After 13 weeks, left and right ventricular weights were 39 and 36% higher than in SED. Left ventricular myocytes were 13% longer, whereas width remained unchanged. After 4 weeks training, myocyte contractility was approximately 20% higher in TR. Peak systolic intracellular Ca²⁺ and time for the decay from systole were 20–35 and 12–17% lower, respectively. These results suggest that increased myofilament Ca²⁺ sensitivity is the dominant effect responsible for enhanced myocyte contractility in TR. Intracellular pH progressively decreased as stimulation frequency was increased in the SED group. This decrease was markedly attenuated in TR and the intracellular pH was significantly higher in the TR group at a stimulation rate of 5–10 Hz. This effect may contribute to the increased contractility observed at the higher stimulation frequencies in TR. A higher intrinsic myofilament Ca²⁺ sensitivity was observed in permeabilised myocytes from the TR group under conditions of constant pH and [Ca²⁺]. Western blot analysis indicated 21 and 46% higher myocardial SERCA-2 and phospholamban, but unaltered Na⁺/Ca²⁺-exchanger levels. Competitive RT-PCR revealed that TR significantly increased Na⁺/H⁺-exchanger mRNA. Conclusion: Intensity controlled interval training increases cardiomyocyte contractility. Higher myofilament Ca²⁺-sensitivity, and enhanced Ca²⁺-handling and pH-regulation are putative mechanisms. Our results suggest that physical exercise induces adaptive hypertrophy in cardiac myocytes with improved contractile function. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Calcium (cellular); Contractile function; Gene expression; Oxygen consumption; Ultrasound

1. Introduction

Recent studies [1,2] suggest that increased aerobic capacity improves quality of life and reduces mortality in heart failure patients. In aerobic endurance training, myocardial enlargement occurs accompanied by enhanced contractile function. However, data on cellular, molecular and integrative mechanisms of exercise-induced adaptation are sparse and sometimes equivocal. In young female rats, treadmill running increases low-affinity Ca²⁺-binding sites on sarcolemma (SL) [3], alters SL Na⁺/Ca²⁺-exchanger activity [4], and increases dihydropyridine-binding capacity of heart homogenates and SL fractions. In old male rats, treadmill exercise enhances sarcoplasmatic reticulum Ca²⁺-ATPase (SERCA-2) gene expression [5] and increases calcium transport by cardiac SERCA-2 [6]. Higher SR Ca²⁺ uptake [7] and SERCA-2 mRNA levels [8] have also
been observed in hearts from swim-trained rats. In contrast, endurance training has been reported to cause no modification in heart SR Ca\(^{2+}\)-uptake and SERCA-2 activity [9], SL Na\(^{+}/Ca\(^{2+}\) exchange activity, ryanodine receptor density or modulation by Ca\(^{2+}\) [10,11], or L-type channel number or intrinsic function [12]. Myocardial contractile function and intracellular Ca\(^{2+}\)-regulation during training was first reported by Laughlin et al. [13] who concluded that endurance training affected neither myocyte shortening characteristics nor Ca\(^{2+}\) dynamics during electrical stimulation at 0.2 Hz, (23°C) in rats. In contrast, Moore et al. [14] found increased shortening at 0.067 Hz, but not at 0.2 Hz (28°C) in another rat model. Palmer et al. [15] reported that myocyte shortening at 0.5 Hz (29°C) was unaltered after training but found a tendency to higher diastolic and peak systolic [Ca\(^{2+}\)] in myocytes from trained hearts. Furthermore, the intracellular [Ca\(^{2+}\)] signal in response to caffeine was altered further suggesting altered myocyte Ca\(^{2+}\) handling. Moore et al. [14] found increased Ca\(^{2+}\)-clearance rate in myocytes from trained hearts, yet the temporal characteristics of myocyte contraction seems to be unaffected by training [13–15].

The aim of this study was to determine the effects of training on cardiomyocyte function in a recently developed model of intensity-controlled interval treadmill running in adult female rats. The regimen induces cardiovascular changes similar to those observed in humans [16], including the largest increase in VO\(_{2\text{max}}\) and heart weight published so far, improved work economy, and reduced resting heart rate. The effect of training on in vivo cardiac performance was assessed by echocardiography. Measurements of contractility, intracellular [Ca\(^{2+}\)] and pH were performed at 37°C and at a range of stimulation rates including physiological values (2–10 Hz).

2. Methods

2.1. Study population

A total of 72 adult female 300–325-g Sprague–Dawley rats (Møllegaards, Denmark) were maintained six in each cage. Light was controlled on a 12-h dark/light cycle. Temperature was 22.5±1.4 and humidity 55.6±4.0%. Animals were fed a pellet rodent diet ad libitum and had free access to water. None of the rats were excluded from the study because they avoided running. The experimental procedures conform to the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes, and the protocol was approved by the Norwegian Council for Animal Research.

2.2. Oxygen uptake and heart rate

VO\(_{2\text{max}}\) and maximal heart rates were measured as previously described [16]. Briefly, after a 15-min warm-up at 40–50% of VO\(_{2\text{max}}\), treadmill speed was increased by 0.03 m/sek every 2 min until observing a leveling-off of oxygen uptake despite increased workload. After completion of VO\(_{2\text{max}}\) test, the rat ran at a work intensity corresponding to 50–60% of VO\(_{2\text{max}}\), followed by a supramaximal intensity run (about 0.06 m/sek above the intensity corresponding to VO\(_{2\text{max}}\)), which lead to exhaustion within about 3 min. The highest heart rate during the supramaximal run was recorded as maximal heart rate. To assess work economy, rats ran 4 min at four fixed submaximal exercise intensities (0.18, 0.21, 0.24 and 0.27 m/sek) before and after the experiment. Based on a previous study [16] all treadmill tests were performed using 25° inclination (47%) of the treadmill. Heart rate was measured by using a frequency modulated acoustical heart rate transmitter operating at approximately 130 kHz [16]. The training and test protocols were performed during the rat’s dark cycle, except for evaluation of basal metabolism and resting heart rates, which were measured in the light cycle when the rats were sleeping [16].

2.3. Training procedure

Trained rats exercised on a treadmill 2 h per day, 5 days per week for 2, 4 or 13 weeks (N=60, n=6). The first group exercised for 4 weeks to study cardiac effects of our exercise model. To study the time course of adaptations, two other groups of rats were trained for 2 or 13 weeks. Additionally, two groups were trained for 4 and 13 weeks to study the intracellular pH regulation in intact myocytes, and myofibrillar responsiveness to Ca\(^{2+}\) and pH in skinned myocytes, respectively.

At the start of every week, VO\(_{2\text{max}}\) was measured and workloads adjusted accordingly. In training rats, exercise intervals alternated between 8 min at 85–90% of VO\(_{2\text{max}}\) and 2 min at 50–60%. Before the first interval, each rat performed a 20-min warm-up at 40–50% of VO\(_{2\text{max}}\). At the day of VO\(_{2\text{max}}\)-testing, trained rats performed eight intervals after the test. In sedentary rats, treadmill running skill was maintained by treadmill running for 15 min at 0° inclination at 0.15 m/sek 3 days per week. After each training or test session, each rat was rewarded with 0.5 g chocolate (Crispo, Nidar Bergene, Norway). Sedentary rats were given the same amount. To study the acute effect of interval training on gene expression of Na\(^{+}/\)H\(^{+}\) exchanger (NHE), six more rats performed a single 2-h interval training session as described above. After the last 8-min interval these rats were immediately removed from the treadmill and anaesthetised with diethyl ether. The heart was quickly removed, cut into right and left ventricle and frozen in liquid nitrogen for later gene analysis of NHE, as described below. For practical reasons it was not feasible to measure the expression of Ca\(^{2+}\)-regulatory proteins in tissue from these animals. The control rats remained at rest.
during the 2-h period. The VO$_{2\text{max}}$ of these ‘acute-rats’ were measured 9 days before the experiment.

2.4. Echocardiography

Echocardiographic examination was performed in animals trained for 13 weeks and corresponding controls. Animals were anaesthetized with 3% halothane (Fluothane, Zeneca, Macclesfield, UK) in a 70% O$_2$, 30% N$_2$, and 0.3% dimethyl sulfoxide (DMSO, Sigma) mixture, intubated and ventilated on a volume controlled ventilator (Model 655, Harvard Apparatus Inc., Edenbridge, UK) with 0.5% halothane in a 70% O$_2$, 30% N$_2$O mixture. Echocardiography was performed with a GE Vingmed Ultrasound System FiV e ultrasound scanner and a 10-MHz linear array probe (GE Vingmed Ultrasound, Horten, Norway). Diastolic and systolic left ventricular wall thickness and cavity diameters were calculated as the mean of measurements in five consecutive cardiac cycles in M-mode long axis recordings.

2.5. Cardiomyocytes isolation

After 2, 4 or 13 weeks of the experimental period, the animals were anaesthetized with diethyl ether and heparinized (0.2 ml heparin 1000 IU/ml i.v.). Hearts were rapidly removed from the animals, kept 1 min in ice-cold perfusion buffer, and connected to an aortic cannula of a standard Langendorff retrograde perfusion system. To balance variation of myocytes isolated, one heart from either group was taken each day. Myocytes were isolated from septal plus left ventricular free wall portions of the myocardium using a previously described protocol [16]. Left and right ventricles were weighed. Samples of left ventricle (~300 mg) was frozen in liquid nitrogen and stored at −80°C for gene analysis. Rest of the left ventricular tissue was processed for cell isolation.

2.6. Fura-2/BCECF loading of isolated myocytes

Myocytes were allowed to stick to laminin-coated coverslips for 1 h in HEPES buffer (37°C, with 5% CO$_2$ and 95% O$_2$, pH 7.4) consisting of (mM) 135 NaCl, 5 KCl, 1 MgCl$_2\cdot$6H$_2$O, 1.8 CaCl$_2\cdot$2H$_2$O, 10 HEPES, 8 glucose-H$_2$O, before gently removing the buffer with loose cells, and quickly adding new HEPES buffer. After a 1–3-h recovery, myocytes were loaded in a dark room at 22±1°C in HEPES buffer containing 2 µM of the acetoxymethyl ester of Fura-2 (Fura-2 AM, Molecular Probes, Eugene, OR) and 0.3% dimethyl sulfoxide (DMSO, Sigma), or 2.0 µM 2',7'-bis(2-carboxethyl)-5(6)carboxy fluorescein acetoxymethyl ester (BCECF-AM) (Molecular Probes) in the pH experiments. After 20 min, cells were washed and maintained for 10 min in Hepes buffer (37°C, pH 7.4) before beginning the experiment.

2.7. Cell shortening and Fura-2 fluorescence

Cells were placed in a cell chamber on an inverted microscope (Diaphot-TMD, Nikon, Tokyo, Japan), and stimulated electrically by bipolar pulses (5-ms duration, 0.1–10 Hz, 37°C) using platinum electrodes on either side of the chamber. During stimulation the cells were superfused at 2 ml/min with HEPES buffer at 37°C. A video camera was attached to the side port of the inverted microscope, and myocytes were analyzed during contraction. The amplitude of cell shortening and the velocity of contraction and relaxation were analyzed from movement of the light/dark contrast of the cell using a video/edge monitor detector (Model 104, Crescent Electronics, Sandy, UT). Signals from the video edge system were digitized and stored in a computer. To examine the time course of contraction we calculated time to peak shortening and time to 50% of peak shortening. Relaxation time was defined as time from maximal shortening to 50% normalization of resting cell length. Time of shortening was defined as the period from the electrical stimulus to peak contraction, or 50% of peak contraction. All measurements were carried out using Fluor ×40 lens (Ph3 DM, Nikon, Japan). The microscope was attached to an ultraviolet excitation light source. A mirror rotating at 500 Hz alternately reflected the ultraviolet excitation light path through band-pass filters of 340 and 380 nm to an optical fiber connected to the microscope. A quadric aperture minimized non-cellular fluorescence. Background fluorescence was measured and subtracted for each cell. Fluorescence emission at 510 nm was counted by a photomultiplier (D-104, PTI, USA). The minimum and maximum fluorescence ratios ($R_{\text{min}}$ and $R_{\text{max}}$) were determined using a protocol described by Frampton et al. [17] using the Ca$^{2+}$ ionophore ionomycin (Sigma, 100 µM). $R_{\text{min}}$ and $R_{\text{max}}$ were similar in cells from SED and TR. As with previous studies (e.g. Ref. [18]) the range of $R_{\text{min}}$ and $R_{\text{max}}$ values represents a much lower dynamic range than that measured in vitro, possibly due to additional fluorescence components from non-cytoplasmic forms of the indicator. Intracellular [Ca$^{2+}$] was calculated assuming a dissociation constant ($K_d$) of 200 nM [19]. This value was confirmed by measuring the $K_d$ for Fura in a limited number of cells (225±28 nM, n=4). To examine the time course of the Ca$^{2+}$ transient we measured peak systolic [Ca$^{2+}$], time to peak [Ca$^{2+}$], time to 50% of peak [Ca$^{2+}$] and time to 50% of the end diastolic [Ca$^{2+}$]. Time course of Ca$^{2+}$ transient was defined as the time period from the electrical stimuli to peak systolic [Ca$^{2+}$], or 50% of peak systolic [Ca$^{2+}$]. From each animal six to 12 cells were studied. All contractility and Ca$^{2+}$ data were calculated from ten consecutive contractions after stabilization at each stimulation frequency. Cells that remained rod shaped, without blebs or other visible morphological alterations, and that responded adequately on electrical stimulation were measured for length and midpoint width. Cellular dimensions
were calculated from 11,271 cells, 170±35 myocytes from each animal.

In animals sacrificed after 4 weeks training, ventricular myocytes were stimulated at 0.1–5 Hz. These experiments demonstrated that differences caused by training occurred at stimulation frequencies above 2 Hz. Therefore, myocytes were stimulated at 2, 5, 7 and 10 Hz in subsequent experiments. After stimulation at 10 Hz, cells were stimulated at 2 Hz for 1 min to ensure that cells were intact. Cells that did not follow at 10 Hz or responded abnormally to the 2-Hz post-stimulation were excluded.

2.8. pH measurements in isolated myocytes

A spinning mirror rotating at 100 Hz alternately reflected the ultraviolet excitation light path through bandpass filters of 440 and 490 nm to an optical fiber connected to the microscope. Background fluorescence was measured and subtracted for each cell. Fluorescence emission at 525 nm was counted by the photomultiplier. After the stimulation protocol, calibration was performed by perfusing cells at pH values of 7.5, 7.0 and 6.5 until a steady fluorescence signal was achieved (3–4 min). The calibration buffers for pH 7.5 and 7.0 consisted of (mM) 140 KCl, 1 MgCl₂, 8 glucose, 10 HEPES and 1 EGTA and 7 µM nigericin. For pH 6.5, Hepes was replaced by MES (10 mM). KOH or HCl was used to adjust pH. In each cell intracellular pH was determined from a linear regression of fluorescence ratio versus the pH values.

2.9. Permeabilised myocytes

Cells were isolated as described above, but Ca²⁺ concentration was kept below 100 nM during and after isolation. After filtering cells through a nylon mesh (250 µm), 1 ml of the cell suspension was centrifuged (600 rpm, 30 s) and the cells resuspended in 2 ml of buffer A containing (mM) 10 EGTA, 100 KCl, 25 HEPES, 5.5 MgCl₂, 5 Na₃ATP, 10 Na₃CrP. To permeabilize myocytes, β-escin (10 µg/ml) was added to buffer A and cells were shaken gently for 30 s, centrifuged (600 rpm, 30 s) and resuspended in buffer A. Cells were placed in the cell chamber on the inverted microscope and perfused with buffer A for 3 min. To study effects of training on myofibrillar responsiveness to Ca²⁺, myocytes were perfused at progressively increasing [Ca²⁺] concentrations (pH 7.0, 37°C) (43, 149, 210, and 310 nM). Progressive increases of [Ca²⁺] caused a progressive shortening of the cell length, which was used as a measure of the myofilament Ca²⁺ sensitivity. To assess the myofilament pH sensitivity at a constant buffered [Ca²⁺] of 210 nM Ca²⁺, the pH was altered from 7.0 to 6.9 and then to 7.1 and the changes in cell length pH were recorded. Equilibrium concentrations of metal ions in the solutions were calculated using a computer program with the affinity constants for H⁺, Ca²⁺ and Mg²⁺ for EGTA taken from Smith and Miller [20]. Affinity constants used for ATP and CrP were those quoted by Fabiato and Fabiato [21]. Corrections for ionic strength, details of pH measurement, allowance for EGTA purity and the principles of the calculations are detailed elsewhere [22]. Free Mg²⁺ concentration was 0.9–1 mM in all solutions.

2.10. Electrophoresis, immunoblotting and densitometry

Samples of intraventricular septum were frozen (−80°C) at the time of animal sacrifice. The tissue was subsequently defrosted to 0°C and homogenised in ice cold homogenisation buffer [23]. Protein content was determined using the Coomassie Plus protein assay (Pierce) and BSA (0.1–1 mg/ml) as standard. SDS–PAGE was performed as described by Currie and Smith [23] with either 6% or 14% Tris–glycine gels for SERCA 2/Na–Ca exchanger and PLB detection, respectively, followed by blotting onto a nitro-cellulose membrane (Hybond C, Amersham). Membranes were then exposed to primary antibodies for one of the three specific proteins under study. SERCA2a: the primary antibody was mouse anti-SERCA2 ATPase monoclonal antibody (IgG₁, Affinity Bioreagents, 1:4000). The secondary antibody was goat anti-mouse IgG–horseradish peroxidase conjugate (Transduction Laboratories, 1:2000). The secondary antibody was goat anti-mouse IgG–horse-radish peroxidase conjugate (Transduction Laboratories, 1:2000). PLB: the primary antibody was used mouse anti-SERCA2 ATPase monoclonal antibody (IgG₁, Affinity Bioreagents, 1:4000). The secondary antibody was goat anti-mouse IgG–horseradish peroxidase conjugate (Transduction Laboratories, 1:2000). Na⁺/Ca²⁺-exchanger: the primary antibody used was polyclonal antisera raise against canine sarcolemmal Na⁺/Ca²⁺ exchanger (Swant, Switzerland, 1:1000). The secondary antibody was goat anti-rabbit–horseradish peroxidase conjugate (Transduction Laboratories, 1:2000). Protein abundance was quantified by scanning developed immunoblots containing known amounts of total homogenate protein. Background was subtracted by scanning equivalent sized areas of nitrocellulose that did not contain immunoreactive protein. Measurements were triplicate and average densitometric measurement was taken over the linear range of protein loading (5–25 µg). An example of the approximately linear relationship between optical density and protein load is shown for a SERCA2 immunoblot in Fig. 5B, similar relationships were constructed for PLB and Na⁺/Ca²⁺ exchanger Western blots.

2.11. Competitive reverse transcriptase-polymerase chain reaction (RT-PCR)

Myocardial mRNA was isolated with Dynabeads Oligo (dT)$_{25}$ (Dynal AS, Oslo, Norway) as previously described [24]. Competitive RT-PCR was performed in a Perkin-Elmer GeneAmp 2400 PCR system using rTth DNA polymerase (Perkin Elmer/Roche Molecular Systems Inc.,
Bronchburg, NJ, USA) according to manufacturers instructions. Reverse transcription for NHE was performed at 50°C for 5 min and 61°C for 40 min followed by 3 min denaturation at 95°C, and PCR amplification and quantified as previously described [24].

2.1.2. Statistical analysis

Data are expressed as mean±S.D. Differences between groups were analyzed with Friedman test for related data and Mann–Whitney U-test for unpaired data, applying appropriate procedures for multiple comparisons [25].

3. Results

3.1. Cardiorespiratory performance

Intensity-controlled interval training significantly increased VO₂max every week, until a plateau 60% above control was reached after 6–8 weeks (Fig. 1A). Maximal heart rate did not change during the training period, whereas resting heart rate in trained animals was 29 and 70 beats/min lower than in SED after 4 and 13 weeks, respectively (Table 1). Work economy, measured as oxygen uptake at a given submaximal running speed, was improved by approximately 13% (S.D.=4.1) after 4 weeks, with no further change at 13 weeks (Fig. 1B). At any given submaximal exercise intensity, heart rate was significantly lower in TR (Fig. 1C). This was also the case after 4 weeks (data not shown). Training consistently increased oxygen pulse, both at rest and during submaximal and maximal exercise (Fig. 1D and Table 1).

3.2. Training induced cardiac hypertrophy

Consistent with previous experiments, the present training protocols induced marked adaptive cardiac hyper-

Fig. 1. (A) Mean±S.D. of maximal oxygen uptake (VO₂max) in the experimental period. Oxygen uptake (B), heart rates (C) and oxygen pulse (D) during treadmill running at fixed submaximal exercise intensities after 13 weeks (data shown individually). * Indicates differences between groups (P<0.001).
<table>
<thead>
<tr>
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<td>637±8.3</td>
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<tr>
<td>2 weeks</td>
<td>642±9.9</td>
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<tr>
<td>4 weeks</td>
<td>390±8.7</td>
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<td>13 weeks</td>
<td>375±9.8</td>
<td>359±7.3**</td>
<td>321±8.6**</td>
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<td>Resting heart rate (beats/min)</td>
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<td>4 weeks</td>
<td>375±9.8</td>
<td>359±7.3**</td>
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<td>13 weeks</td>
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<td>18±2</td>
<td>19±2</td>
<td>20±2</td>
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<td>Resting oxygen pulse (VO₂/HR) (ml/kg/beat)</td>
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<td>4 weeks</td>
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<tr>
<td>13 weeks</td>
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<tr>
<td>Anterior wall thickness (µm)</td>
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<td>Left ventricular mass (mg)</td>
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<td>Training</td>
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<td>Left ventricular cell length (µm)</td>
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*VO₂, oxygen uptake. Differences between groups, *P<0.05; **P<0.01. All group data are mean±S.D. calculated from six rats at 2 weeks and 12 rats at 4 and 13 weeks. Cellular dimensions are calculated from a total of 11,271 cells, 170±35 in each rat.

At 4 weeks, left and right ventricular weights were 12 and 19% higher, respectively, in trained animals than in sedentary controls and at 13 weeks the corresponding increments were 39 and 36% (Table 1). Training for 4 or 13 weeks increased left ventricular myocytes length by 7 and 13%, respectively, whereas width remained unchanged (Table 1). Relative wall thickness assessed by echocardiography was reduced after 13 weeks (Table 2), indicating an eccentric pattern of hypertrophy.

3.3. Myocyte contractility and Ca²⁺-handling

Sample Fura-2 fluorescence measurements (converted to [Ca²⁺]) and the accompanying shortening record are

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**Table 2**

<table>
<thead>
<tr>
<th>Echocardiographic data*</th>
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<tr>
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<tr>
<td>Fractional shortening (%)</td>
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</table>

* Left ventricular size and function in anesthetized rats assessed with M-mode echocardiography after 13 weeks. PWT, posterior wall thickness; LVIR, left ventricular internal radius. Data are mean±S.D. calculated from six rats in each group.
shown in Fig. 2A. Panel (i) shows the averaged signals recorded from a myocyte isolated from a heart from the SED group during stimulation at 7 Hz. Panel (ii) shows the comparable transients recorded after 13 weeks training (TR). At this stimulus rate the Ca\(^{2+}\) transient amplitude and end diastolic [Ca\(^{2+}\)] was considerably lower in the TR group, despite a greater degree of fractional shortening.

The mean values of both Ca\(^{2+}\) transients and cell shortening from a range of stimulus rates are shown in the subsequent panels of Figs. 2B,C and 3A,B). As shown in these figures, myocytes isolated from the TR group displayed a significantly greater fractional shortening than the SED group at stimulus frequencies >2 Hz. The effect was similar after 4 and 13 weeks, with an approximate

Fig. 2. (A) averaged Ca\(^{2+}\) transients and cell shortening recorded at 7-Hz stimulation. Data are calculated from a total of 36 cells, three in each rat (n=6). Relationship between stimulation frequency and maximal shortening (B(i) and B(ii)), intracellular Ca\(^{2+}\) (C(i)), and intracellular pH (C(ii)) at 4 weeks. Note that pH is measured at 2–10 Hz in myocytes from a separate training experiment. Intact TR myocytes become less acidic with increasing stimulation frequencies. Each data point represents mean±S.D. of 62 cells, 9±3 in each rat (n=6). In each cell data were calculated as the mean of ten consecutive contractions after stabilization at each stimulation frequency. * Indicates differences between groups (P<0.01).
Fig. 3. Relationship between stimulation frequency and maximal extent of shortening (A(i) and A(ii)) and intracellular \([\text{Ca}^{2+}]\) (B(i)) at 13 weeks. Panel B(ii) and C represent half time of \(\text{Ca}^{2+}\) decay and cellular relengthening time. Each data point represents mean±S.D. of 62 cells, 9±3 in each rat (\(n=6\)). In each cell data were calculated as the mean of ten consecutive contractions after stabilization at each stimulation frequency. * Indicates differences between groups (\(P<0.001\)).

20% increase in fractional shortening at 2 and 5 Hz in both TR groups. Similar trends were present at lower stimulation rates and after 2 weeks of training (data not shown). \(\text{Ca}^{2+}\)-transients and amplitudes were consistently lower in TR than SED myocytes at all stimulation frequencies (Figs. 2A(i) and 3B(ii)) an effect that was evident after 2 weeks of training (data not shown). These results suggest that training increased the myofilament \(\text{Ca}^{2+}\) sensitivity. A \(\text{Ca}^{2+}\)-sensitivity index (myocyte shortening/\(\text{Ca}^{2+}\)-transient amplitude) was 72.3±24.0% higher in TR (\(P<0.001\)). Both the time to 50% relaxation and the time to 50% decay of the \(\text{Ca}^{2+}\) transient were lower in the TR group (Fig. 3B(ii) and C). The more rapid decrease of intracellular \(\text{Ca}^{2+}\) may reflect increased myofilament \(\text{Ca}^{2+}\)-binding
and/or increased Ca\(^{2+}\)-removal from the cytosol by the SR
Ca\(^{2+}\) pump and/or the sarcolemma Na\(^+\)/Ca\(^{2+}\) exchange. As illustrated in Fig. 5 and shown in Table 3, Western analysis demonstrated approximately 25% up-regulation of SERCA-2 and PLB in TR hearts but no significant changes in the abundance of Na\(^+\)/Ca\(^{2+}\)-exchanger.

### 3.4. Intracellular pH measurements

In the sedentary group steady state intracellular pH decreased with increasing stimulation rates (Fig. 2C(ii)). At 5 Hz, intracellular pH was approximately 0.15 units more acid than at 1 Hz. This was in contrast to the TR group, where no significant shift was observed. Thus intracellular pH was significantly less acid in the TR than in SED at 5–10 Hz stimulation, which could contribute to the enhanced contractility. This difference between experimental groups may arise from reduced acid production and/or increased capacity for intracellular pH regulation. As shown in Table 3 NHE expression was up-regulated in LV tissue from trained rats both acutely (130%) and at 2 weeks (20%). Similar trends were observed at 4 and 13 weeks.

### 3.5. Myofilament Ca\(^{2+}\)-sensitivity in intact and permeabilised myocytes

At end-diastole the rate of change of [Ca\(^{2+}\)] and cell shortening are minimal, thus these values can be used to assess myofilament Ca\(^{2+}\) sensitivity in the intact cell. Fig. 4A demonstrates a significantly greater cell shortening at comparable [Ca\(^{2+}\)] in the TR group suggesting increased myofilament sensitivity. This was investigated more directly by permeabilising single cardiac myocytes using betaescin and perfusing with a range of [Ca\(^{2+}\)] in the presence of 10 mM EGTA. Fig. 4B illustrates a typical shortening record from a permeabilised cardiac myocyte. As shown in Fig. 4C and D higher myofilament Ca\(^{2+}\)-sensitivity in the TR group was confirmed by the experiments in permeabilised myocytes. Furthermore, acid and alkaline shifts in pH at a set [Ca\(^{2+}\)] (210 nM) produced larger changes in cell shortening in SED suggesting that the myofilaments in TR are less sensitive to altered pH (\(P<0.01\)).

![Fig. 4](https://example.com/fig4.png)

Fig. 4. In rats trained for 13 weeks a larger extent of shortening with increasing simulation frequencies indicates increased sensitivity to Ca\(^{2+}\) (A). Panel B shows a typical cell length record from a permeabilised myocyte exposed to a range of [Ca\(^{2+}\)] and bathing pH. After 13 weeks of endurance training skinned myocytes responded more to changes in Ca\(^{2+}\) (C), and were less affected by changes in pH compared to SED (D). In intact cells each data point represents mean±S.D. of 62 cells, 9±3 in each rat (n=6). In each cell data are calculated as the mean of ten consecutive contractions after stabilization at each stimulation frequency. In skinned cells, data were calculated after stabilization at each [Ca\(^{2+}\)] and pH in 64 cells, 9±3 in each rat (n=6). * Indicates differences between groups (\(P<0.01\)).
4. Discussion

In the present study, intensity controlled interval training induced larger changes in cardiorespiratory function, myocardial contractility and cardiomyocyte calcium handling than previously reported training regimens. A major finding was consistently increased myocyte contractile function and calcium sensitivity. Evidence is presented to indicate that several aspects of myocyte function are altered in the exercise group. How these changes may contribute to the positive inotropic effect is discussed.

4.1. Cardiorespiratory performance

Animals trained and tested according to the present procedures [16] displayed most of the cardiorespiratory

Table 3
Biochemical data^a

<table>
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<tr>
<th></th>
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<th>2 weeks</th>
<th>4 weeks</th>
<th>13 weeks</th>
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<td><strong>Competitive RT-PCR</strong></td>
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<td>Sodium hydrogen exchanger (amol cDNA/g tissue)</td>
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<td>Sedentary</td>
<td>390.0±95.0</td>
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<td>Training</td>
<td>896.7±47.9**</td>
<td>202.0±43.9*</td>
<td>358.4±77.6</td>
<td>235.3±108.2</td>
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<tr>
<td><strong>Western blot</strong></td>
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<tr>
<td>Sarcoplasmatic reticulum Ca^{2+}-ATPase-2 (μg/mg)</td>
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<tr>
<td>Sedentary</td>
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<td>1.03±0.06*</td>
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<td>Phospholamban (μg/mg)</td>
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<td>Sodium calcium exchanger (μg/mg)</td>
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^a Data from acute training and 2 weeks are mean±S.D. calculated from six rats in each group, whereas there were 12 rats in each group at 4 and 13 weeks. Differences between groups, *P<0.05; **P<0.01.
changes observed in humans. The increases in VO$_{2\text{max}}$, heart weights, oxygen pulse and the reduction in heart rate were larger than those previously reported (e.g. Ref. [12]). This is likely to result from the high aerobic intensity of the training regimen. A major part of the changes occurred within 4 weeks and VO$_{2\text{max}}$ plateaued after about 6 weeks of endurance training, with the majority of changes within 4 weeks. This corresponds with the changes in myocyte contractility and Ca$^{2+}$-handling (see below).

4.2. Echocardiography and post-mortem data

These measurements revealed significant decreases in wall thickness in the hearts from the TR group (13 weeks). There were trends to indicate increased internal diameter during diastole, and resting heart rate decreased in the TR group. These results taken together with the post-mortem data on ventricle weights and myocyte dimension suggests that significant cardiac hypertrophy has taken place during training. The decreased wall thickness and increased myocyte length suggests an eccentric form of remodeling.

4.3. Myocyte contractility

This study presents isolated myocyte contractility data obtained at a wide range of stimulus frequencies, with an initial study (4 week training) which used a frequency range of 0.1–5 Hz in line with previous studies [13,14]. The clearest differences in contractility between the two experimental groups emerged at the highest stimulation frequencies. For this reason, in subsequent experiments, isolated cells were stimulated at higher frequencies (2–10 Hz). As shown in Figs. 2 and 3, myocytes isolated from the TR groups shortened to a greater extent and relaxed faster than the comparable SED group. These results are in agreement with data from Moore et al. [14] who found increased extent of shortening in myocytes from trained animals, but conflicts with results of Laughlin et al. [13]. Differences in training protocols, stimulation frequencies and temperature used when stimulating the myocytes might explain these conflicting results. Significant differences between TR and SED were evident at stimulus frequencies above 2 Hz, the positive inotropic effect observed at 4 weeks was not significantly different from that at 13 weeks (compare 2 and 5 Hz values).

4.4. Ca$^{2+}$ handling

In this study, increased myocyte shortening in TR was associated with lower peak systolic intracellular [Ca$^{2+}$]. The effect occurred at all stimulus rates but was most prominent at higher stimulus frequencies. Lower peak systolic Ca$^{2+}$ transients have been reported earlier by Moore et al. [14] but not by others (e.g. Ref. [13]). The reduction in peak systolic Ca$^{2+}$ concentration in TR myocytes could be due to: (i) reduced Ca$^{2+}$ released into cytosol via sarcolemma and SR; (ii) dilution of released Ca$^{2+}$ into the sarcoplasm due to increased average myocyte volume; (iii) increased intracellular Ca$^{2+}$ buffering capacity. The first two possibilities are unlikely since reduced Ca$^{2+}$ influx or diluted cytosolic [Ca$^{2+}$] would reduce the Ca$^{2+}$ binding to myofilaments and reduce contractility. The final possibility is feasible since only a small fraction of Ca$^{2+}$ that is released into and removed from the sarcoplasm during an excitation–contraction coupling cycle exists as free Ca$^{2+}$ [26]. This adaptation to training is consistent with lower diastolic and systolic [Ca$^{2+}$] in TR myocytes. Tibbits et al. [27] have demonstrated that Ca$^{2+}$ binding sites increased by about 65% in papillary muscle from trained rats. Penpargkul et al. [28] reported enhanced Ca$^{2+}$-binding by cardiac SR from trained rats. Lower diastolic [Ca$^{2+}$] in TR myocytes could also result from enhanced SL ATP-dependent Ca$^{2+}$ extrusion [29] and/or mitochondrial metabolism [30], thus effectively lowering the set point for Ca$^{2+}$ regulation [31] in trained myocytes. Changes in myofilament Ca$^{2+}$ affinity can dramatically affect amplitude and time course of the Ca$^{2+}$ transient. Sulmazole increases myofilament Ca$^{2+}$ binding affinity and peak myocardial force development, reduces peak systolic Ca$^{2+}$ [32] and increases Ca$^{2+}$ transient decay. Similarly, intracellular alkalosis increases myocyte shortening by increasing myofilament Ca$^{2+}$ sensitivity. The accompanying Ca$^{2+}$ transient is smaller in amplitude and shorter in duration [33]. As discussed below, TR myocytes have significantly less acidic intracellular pH at high stimulus rates (>2 Hz). It is possible that the lower systolic [Ca$^{2+}$] in the TR group is due to higher intracellular pH. However, this explanation is insufficient since intracellular pH is comparable below 2 Hz, yet TR myocytes shorten to a greater extent. Without data on intracellular Ca$^{2+}$ buffering capacities or Ca$^{2+}$ flux, free [Ca$^{2+}$] cannot be directly related to the amount of Ca$^{2+}$ released into the cytosol. In the present study TR increased the expression of SERCA2 and PLB. Thus increased Ca$^{2+}$ uptake capacity of the SR due to increased SERCA2 expression could account for the increased rate of decay of the Ca$^{2+}$ transient. Up-regulation of SERCA2 has been previously reported to have a positive inotropic effect by increasing the Ca$^{2+}$ content of the SR [34], which in the absence of any other changes would increase SR Ca$^{2+}$ release, peak systolic [Ca$^{2+}$] and contractility. Increased Na$^+$/Ca$^{2+}$-exchanger levels have been observed in some models of hypertrophy [35]. However, no changes were observed in the present study, which is in agree with some studies [36] but in contrast to others [4]. In summary, data suggests that the dominant factor for altered Ca$^{2+}$ handling is increased myofilament Ca$^{2+}$ binding, as possible increases in SR Ca$^{2+}$ released could not be distinguished.

4.5. Effects of training on intracellular pH

This is the first study to show that TR myocytes
becomes less acidic at physiological stimulation frequencies compared to SED. In both groups increasing stimulation frequency decreased intracellular pH. However, in the TR group the acid shift was significantly less than that seen in the SED group. Similar shifts to that seen in the SED have been reported for other cardiac preparations [37]. This and subsequent studies indicated that the acidic shift in pH correlated with the rise of intracellular $[Ca^{2+}]$ and was dependent on glycolysis. Whether the difference between the TR and SED groups is due to increased pH buffer capacity, reduced intracellular $[Ca^{2+}]$ or increased amount and/or activity of proteins involved in intracellular pH regulation has yet to be defined. As seen in Table 3, NHE mRNA was markedly higher in TR (130%) immediately after exercise cessation. A transient increase in NHE mRNA expression at 2 weeks was observed in the TR group and a trend toward increase also measured at 4 and 13 weeks. However, the resolution of these measurements was poor, the large inter-sample variation prevented the resolution of ~20% changes in NHE expression.

### 4.6. Myofilament $Ca^{2+}$ sensitivity

In line with Moore et al. [14], the $Ca^{2+}$ and shortening data in intact and permeabilised myocytes suggest a training induced increase in the sensitivity of the contractile element to $Ca^{2+}$. Part of the increased $Ca^{2+}$ sensitivity observed in intact myocytes can be attributed to the higher intracellular pH observed at physiological stimulation frequencies. However, permeabilised cells from the TR group shortened to a greater extent than SED myocytes in the presence of a constant buffered pH. These results would indicate that the contractile proteins of the myocytes from the TR group had an intrinsically higher $Ca^{2+}$ sensitivity than the SED. These measurements cannot distinguish between an altered affinity of the myofilaments for $Ca^{2+}$ and altered maximal force capacity, furthermore, shortening is also sensitive to the passive resistive element in cardiac myocytes. Therefore, further work is required to characterize this result. An interesting subsidiary observation from this aspect of the study was the response of permeabilised cells to altered pH at constant $[Ca^{2+}]$ (Fig. 4D). As previously reported (reviewed in Ref. [38]), acid pH decreased and alkaline pH increased myofilament shortening in myocytes from SED and TR groups. In an analogous way to intracellular $[Ca^{2+}]$, this indicates that a component of the enhanced myocyte contractility could be attributed to the more alkaline intracellular pH in the TR group at high stimulus frequencies. However, since enhanced shortening is observed at stimulus rates with comparable intracellular pH (2 Hz or less), then the altered intracellular pH cannot be the complete explanation. The results shown in Fig. 4D also suggest that the sensitivity of the myofilaments to pH is less in the TR group. Further work is required to fully characterize this effect, but altered pH sensitivity may accompany the altered $Ca^{2+}$ sensitivity of the myofilaments in the TR model. Previous work has shown changes in the expression of troponin I isoforms during perinatal development and stress in cardiac muscle [39]. Different troponin I isoforms are known to regulate the pH sensitivity of cardiac myofilaments [40].

### 4.7. Relevance for heart failure studies

The present study and others [6] suggest that training improves myocardial function in heart failure by restoring the level of calcium cycling proteins and myofilament sensitivity. As shown in Table 4, the changes in SERCA2, PLB, time course of the $Ca^{2+}$ transient, myofilament $Ca^{2+}$ sensitivity, and cell shortening are the converse of those in myocytes from failing hearts after myocardial infarction. The pattern of change in the rat closely resembles those observed in human heart failure, except for the diverging experimental data on the Na±Ca±exchanger [35,41]. It is not known whether this potential discrepancy reflects a physiologically important difference between the human and the rat species, or results from methodological characteristics, such as experimental model, infarct size, degree and duration of heart failure, and the fact that mRNA and protein levels do not always change at the same time and in the same manner [35]. Until myocardial tissue from trained humans becomes available, it is likely that most data on calcium cycling proteins and calcium sensitivity will come from experimental models.

### 4.8. Conclusion

Intensity controlled interval training increases car-

<table>
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<th>Protein</th>
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<td></td>
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<td>SERCA2</td>
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</tr>
<tr>
<td>PLB</td>
<td>Normal or decreased</td>
<td>Normal or decreased</td>
</tr>
<tr>
<td>Ryanodine receptor</td>
<td>Normal or decreased</td>
<td>Normal or decreased</td>
</tr>
<tr>
<td>NCX</td>
<td>Increased</td>
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<td>L-type calcium channels</td>
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<td>Normal or decreased</td>
</tr>
<tr>
<td>Myofilament $Ca^{2+}$ sensitivity</td>
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</tr>
<tr>
<td>Cardiomyocyte shortening</td>
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<td>Normal or decreased</td>
</tr>
</tbody>
</table>

Table 4
Calcium cycling proteins and myofilament sensitivity (reviewed in Refs. [35,41,42])
diomyocyte contractility. Higher myofilament Ca$^{2+}$-sensitivity, and enhanced Cu$^{2+}$-handling and pH-regulation are putative mechanisms. Thus, physical exercise induces adaptive hypertrophy in cardiac myocytes, and improves myocyte contractile function.

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