Calcium homeostasis in rat cardiomyocytes during chronic hypoxia: a time course study

Jian-Ming Pei,* Gennadi M. Kravtsov,* Song Wu, Rapti Das, Man Lung Fung, and Tak Ming Wong. Calcium homeostasis in rat cardiomyocytes during chronic hypoxia: a time course study. Am J Physiol Cell Physiol 285: C1420–C1428, 2003; 10.1152/ajpcell.00534.2002.—The present study determined Ca\(^{2+}\) handling in the hearts of rats subjected to chronic hypoxia (CH). Spectrofluorometry was used to measure intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\),i]c) and its responses to electrical stimulation, caffeine, and isoproterenol in myocytes from the right ventricle of rats breathing 10% oxygen for 1, 3, 7, 14, 21, 28, and 56 days and age-matched controls. The protein expression of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) and its ryanodine receptor (RyR) were measured. The uptake of 45Ca\(^{2+}\) by SERCA, release by RyR, and extrusion by Na\(^+/Ca^{2+}\) exchange (NCX) were determined. It was found that Ca\(^{2+}\) homeostasis and Ca\(^{2+}\) responses to β-adrenoceptor stimulation reached a new equilibrium after 4 wk of CH. Ca\(^{2+}\) content in the sarcoplasmic reticulum (SR) was reduced, but cytosolic Ca\(^{2+}\) remained unchanged after CH. Expression of SERCA and its Ca\(^{2+}\) uptake, Ca\(^{2+}\) release via RyR, and NCX activity were suppressed by CH. The results indicate impaired Ca\(^{2+}\) handling, which may be responsible for the attenuated Ca\(^{2+}\) responses to β-adrenoceptor stimulation in CH.

intracellular calcium ion concentration; calcium-adenosine-triphosphatase; ryanodine receptor; sodium/calcium exchange; sarcoplasmic reticulum; β-adrenoceptor; chronic hypoxia

THE SARCOLEMMA and sarcoplasmic reticulum (SR) of the cardiomyocyte play an important role in Ca\(^{2+}\) homeostasis in the heart. Electrical stimulation of cardiomyocytes evokes depolarization of the sarcolemma, leading to Ca\(^{2+}\) influx through the voltage-gated Ca\(^{2+}\) channels (4, 12, 33). A small rise in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\),i]) in the narrow space between the internal surface and the SR triggers the opening of the ryanodine receptor (RyR), resulting in an efflux of Ca\(^{2+}\) from SR into the cytoplasm (7, 36). This sudden elevation of [Ca\(^{2+}\),i], which is transient, is required for the development of contractile activity (14). In the rat cardiomyocyte, immediately after contraction >90% of the Ca\(^{2+}\) is sequestered back to the SR via sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) and close to 7% is pumped out of the cell via Na\(^+/Ca^{2+}\) exchange (NCX) (5, 32). It is well established that Ca\(^{2+}\) handling is essential for many cardiac activities, including excitation-contraction coupling. Therefore, investigation of Ca\(^{2+}\) handling in different pathological situations such as chronic hypoxia (CH) will further our understanding of the etiology of impaired cardiac functions.

Recently, we showed (27) that the electrically induced [Ca\(^{2+}\),i] transient of cardiomyocytes from rats subjected to 28 days of CH was reduced. Furthermore, the elevation in electrically induced [Ca\(^{2+}\),i] transient to β-adrenoceptor (β-AR) stimulation in the same cardiomyocytes was also attenuated (26). The reduction in the electrically induced [Ca\(^{2+}\),i] transient in CH may result from impaired Ca\(^{2+}\) handling by both the sarcolemma and the SR. To test this hypothesis we determined the activity and expression of the SR proteins SERCA and RyR, which are involved in handling of Ca\(^{2+}\), and the activity of NCX in the sarcolemma of ventricular myocytes from the right heart of rats subjected to CH for 1 to 2 mo. The changes were correlated with changes in [Ca\(^{2+}\),i], transients induced by electrical stimulation and caffeine. In addition, we correlated the time course of changes in Ca\(^{2+}\) handling with that of the Ca\(^{2+}\) response to isoproterenol. The results showed that expression of SERCA2 protein, Ca\(^{2+}\) uptake via SERCA, Ca\(^{2+}\) release via RyR, Ca\(^{2+}\) extrusion via NCX, and [Ca\(^{2+}\),i] transients induced by electrical stimulation and caffeine were suppressed after CH. The altered handling of Ca\(^{2+}\) by SR and sarcolemmal membrane correlated well with the attenuated Ca\(^{2+}\) responses to β-AR stimulation. Four weeks is required for full adaptation to hypoxia.

MATERIALS AND METHODS

All procedures in this study were approved by the Committee on the Use of Live Animals in Teaching and Research at the University of Hong Kong.

Chronic hypoxia. Male Sprague-Dawley rats that weighed 100–150 g at the start of the experiment were randomly divided into two groups. One group was exposed to CH, and the control group was maintained in room air. All rats were kept in the same room with the same light-dark cycle. For CH, rats were given inspired oxygen (10% O\(_2\)) in a 300-liter advertisement

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acrylic chamber. The hypoxic environment was established with the inflow of a mixture of room air and nitrogen that was regulated by an oxygen analyzer (model 175518A, Gold Edition, Vacuum Med; Refs. 2, 28, 29). CO₂ was absorbed by soda lime granules, and excess humidity was removed by a desiccator. Temperature was maintained at 19–21°C. The chamber was opened twice a week for 1 h to clean the cages and replenish food and water. rats were exposed to hypoxia for 1, 3, 7, 14, 21, 28, or 56 days. Experiments were performed immediately after removal of the rats from the chamber. The rats were decapitated, and the hearts were quickly removed. The heart and body weights were obtained; the hearts exhibited hypertrophy after 28 and 56 days of hypoxia (Table 1). The PO₂ of arterial blood in the chronically hypoxic rats was reduced by 35% compared with control rats.

**Isolation of ventricular myocytes.** Ventricular myocytes were isolated from the right heart with a collagenase perfusion method described previously (10). Immediately after decapitation, the hearts were rapidly removed and perfused in a retrograde manner at a constant flow rate (10 ml/min) with an oxygenated Joklik-modified Eagle’s medium supplemented with 1.25 mM CaCl₂ and 10 mM HEPES, pH 7.2, at 37°C for 5 min. This was followed by 5-min perfusion with the same medium without Ca²⁺. Collagenase was then added to the medium to a concentration of 125 U/ml with 0.1% (wt/vol) bovine serum albumin (BSA). After 35–45 min of perfusion with the medium containing collagenase, the atria were discarded. The right ventricle tissues were dissociated by shaking in the same oxygenated collagenase-free solution for 5 min at 37°C. Ventricular tissues were cut into small pieces with a pair of scissors followed by stirring with a glass rod for 5 min. The procedure separated the ventricular myocytes. The residue was filtered, centrifuged at 100 g for 1 min, and resuspended in fresh Joklik solution with 1% BSA. More than 70% of the cells were rod shaped and impermeable to Trypan blue. The Ca²⁺ concentration of the Joklik solution was increased gradually to 1.25 mM over 40 min.

**Measurement of [Ca²⁺]**. Myocytes were incubated for 30 min with 5 μM fura 2-AM in Joklik solution supplemented with 1.25 mM CaCl₂. The unincorporated dye was removed by washing the cells twice in fresh incubation solution. The loaded cells were kept at room temperature (25°C) for 30 min before measurement of [Ca²⁺]., to allow the fura 2-AM in the cytosol to deesterify. A low concentration of fura 2-AM (5 μM) was loaded at a relatively low temperature of 25°C to minimize the effects of compartmentalization of the esters (10).

The ventricular myocytes loaded with fura 2-AM were transferred to the stage of an inverted microscope (Nikon) in a superfusion chamber at room temperature. The inverted microscope was coupled to a dual-wavelength excitation spectrofluorometer (Photo Technical International, South Brunswick, NJ). The myocytes were superfused with a Krebs bicarbonate buffer containing (in mM) 118 NaCl, 5 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 1.25 CaCl₂, 25 NaHCO₃, and 11 glucose, with 1% dialyzed BSA and a gas phase of 95% O₂-5% CO₂, pH 7.4. The myocytes selected for the study were rod shaped and quiescent with clear striations. They exhibited a synchronous contraction (twitch) in response to suprathreshold 4-ms stimuli at 0.2 Hz delivered by a stimulator (Grass S88) through two platinum field-stimulation electrodes in the bathing fluid. A transient rise of [Ca²⁺]i, is associated with each contraction of a cardiac muscle cell, and this is widely termed the “[Ca²⁺]i transient” (37). In many mammalian species including the rat, the [Ca²⁺]i transient is mainly caused by release of Ca²⁺ from the SR into the cytoplasm triggered by Ca²⁺ influx via the L-type Ca²⁺ channel on membrane depolarization, and it is this rise of [Ca²⁺]i that is accompanied by cell shortening (40). The caffeine-induced [Ca²⁺]i transient is an index of the Ca²⁺ content in the SR because caffeine depletes the SR of Ca²⁺ (18, 19, 31, 35). In the present study, we measured the amplitude and decay of both electrically induced and caffeine-induced [Ca²⁺]i transients. Fluorescent signals obtained at 340-nm (F₃₄₀) and 380-nm (F₃₈₀) excitation wavelengths were stored in a computer for data processing and analysis. The fluorescence ratio F₃₄₀/F₃₈₀ was used to represent [Ca²⁺]i changes in the myocyte. In some experiments, the resting [Ca²⁺]i was measured while the electrical stimulation was off.

To measure the decay rate (τ) of the [Ca²⁺]i transients, we determined the τ value according to the equation [Ca²⁺]i = [Ca²⁺]peak × e⁻τt + [Ca²⁺]baseline, where τ represents the continuous variable of time. The decay of the electrically induced [Ca²⁺]i transient indicates the uptake of Ca²⁺ by SR (3, 13, 15, 17, 18, 23). The decay of the caffeine-induced [Ca²⁺]i transient reflects NCX activity because caffeine keeps the RyR open and therefore the decay in Ca²⁺ is due to NCX activity rather than reuptake by SR (18, 31, 35).

**Isolation of SR and measurement of ⁴⁵Ca²⁺ uptake.** SR vesicles were obtained by a method described previously (34, 42) with some modifications. Briefly, freshly isolated right ventricles were washed in ice-cold 0.9% NaCl and homogenized in an extraction medium containing (in mM) 15 Tris-HCl, 10 NaHCO₃, 5 NaN₃, 250 sucrose, and 1 EDTA (2°C; pH 7.0; 5 ml/g tissue) with a Polytron PT 35 homogenizer. The homogenate was centrifuged for 5 min at 3,000 g to remove cellular debris. The supernatant was further centrifuged at 48,000 g for 75 min, and the supernatant was discarded. The pellet was suspended in 5 ml of a mixture of 0.6 M KCl and 20 mM Tris-HCl (pH 7.0) and centrifuged at 48,000 g for 60 min. The final pellet was rehomogenized in 1 ml of 250 mM sucrose and 40 mM imidazole-HCl with a Potter-Elvehjem homogenizer with a Teflon pestle and stored at ~70°C. All solutions contained three protease inhibitors: soybean trypsin inhibitor (40 μg/ml), 0.1% phenylmethylsulfonyl fluoride (PMSF), and leupeptin (0.5 μg/ml).

### Table 1. Effects of chronic hypoxia on body weight and heart weight in rats

<table>
<thead>
<tr>
<th>Groups, days</th>
<th>BW, g</th>
<th>HW, mg</th>
<th>HW/BW, mg/g</th>
<th>BW, g</th>
<th>HW, mg</th>
<th>HW/BW, mg/g</th>
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<tr>
<td>Normoxic</td>
<td>1</td>
<td>289.5 ± 13.44</td>
<td>1232.4 ± 73.54</td>
<td>4.13 ± 0.8</td>
<td>296.5 ± 14.85</td>
<td>1222.5 ± 62.93</td>
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<td>7</td>
<td>289.5 ± 16.13</td>
<td>1198.2 ± 104.42</td>
<td>4.05 ± 0.3</td>
<td>282.3 ± 8.74</td>
<td>1186.1 ± 84.79</td>
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<tr>
<td></td>
<td>14</td>
<td>289.5 ± 14.43</td>
<td>1220.8 ± 123.63</td>
<td>4.10 ± 0.7</td>
<td>257.4 ± 14.89</td>
<td>1122.7 ± 184.93</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>289.5 ± 17.14</td>
<td>1223.3 ± 128.68</td>
<td>4.10 ± 0.6</td>
<td>239.7 ± 19.86</td>
<td>1108.2 ± 182.26</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>304.8 ± 30.22</td>
<td>1242.3 ± 117.41</td>
<td>4.07 ± 0.6</td>
<td>213.1 ± 19.23</td>
<td>1126.3 ± 186.42</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>455.6 ± 31.51</td>
<td>1453.7 ± 272.65</td>
<td>3.19 ± 0.4</td>
<td>283.7 ± 17.06</td>
<td>1503.5 ± 193.34</td>
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</table>

Values are means ± SE; n = 15 rats in each group. BW, body weight; HW, heart weight. *P < 0.01 vs. corresponding normoxic control.
The ATP-dependent transport of Ca\(^{2+}\) to SR was measured at room temperature (22\(^\circ\)C) with a method described previously (22). SR protein (50–100 \(\mu\)g) was added to 1 ml of a medium that contained 40 mM imidazole-HCl (pH 7.0), 100 mM KCl, 20 mM NaCl, 5 mM MgCl\(_2\), 4 mM ATP-Н\(_2\), 1.3 \(\mu\)M 45CaCl\(_2\), 5 \(\mu\)M Ru-360, an inhibitor of Ca\(^{2+}\) uptake in mitochondria (38), and 5 \(\mu\)M calmidazolium, an inhibitor of sarcoclemmal Ca\(^{2+}\)-ATPase (24). The concentration of free Ca\(^{2+}\) in this solution (5 \(\mu\)M) was determined by a Ca\(^{2+}\)-EGTA buffer and calculated according to Fabiato and Fabiato (11). To measure the oxalate-supported Ca\(^{2+}\) uptake, 5 mM K-oxalate was added to the aforementioned solution. After 2–20 min, aliquots of 0.9 ml were filtered through Millipore filters (0.45 \(\mu\)m; Bedford, MA). Filters were washed three times with 4 ml of cold (2–4\(^\circ\)C) solution containing 40 mM imidazole-HCl (pH 7.0), 100 mM KCl, and 0.1 mM EGTA.

Figure LA shows that the 45Ca\(^{2+}\) uptake was linear for 5 and 10 min in the absence and presence, respectively, of 5 mM K-oxalate. Therefore, measurement of the rate of uptake was made close to the end of the linear phase, 4 and 9 min in the absence and presence, respectively, of K-oxalate. The ATP-dependent transport of Ca\(^{2+}\) by SERCA was defined as the difference between the rate of 45Ca\(^{2+}\) uptake in a K-oxalate-containing solution in the presence and absence of 5 \(\mu\)M cyclopiazonic acid (CPA), a specific inhibitor of SERCA (Ref. 30; Fig. 1B). The difference in uptake in the presence and absence of 20 \(\mu\)M ryanodine, a special blocker of RyR, was defined as the Ca\(^{2+}\) release via the RyR receptor (Fig. 1C).

Plasma membrane purification and NCX assay. For purification of plasma membrane vesicles, the procedure described by Hanf et al. (16) was used with slight modification. Briefly, 1 g of ventricular tissue was washed in ice-cold 0.9% NaCl and homogenized in ice-cold buffer containing 0.6 M sucrose and 10 mM imidazole-HCl (pH 7.0). The homogenization consisted of two bursts of 7 s each at half-maximum speed with a Polytron PT 35. The homogenate was centrifuged at 1,000 \(\times\) g for 10 min. The supernatant was then centrifuged at 160,000 \(\times\) g for 90 min. The pellet representing the sarcolemma-enriched fraction was dissolved in 0.5 ml of solution A (in mM: 100 NaCl, 50 LiCl, 6 KCl, 20 HEPES-Tris, pH 7.4) and assayed for NCX activity. All solutions contained all three protease inhibitors, soybean trypsin inhibitor (40 mg/ml), PMSF (0.1%), and leupeptin (0.5 \(\mu\)g/ml).

NCX was estimated as specific Na\(^+-\)dependent Ca\(^{2+}\) uptake following the protocol described previously (16) with some modifications. Briefly, 4 \(\mu\)l of the vesicle suspension was incubated for 50 min at 22\(^\circ\)C to load Na\(^+\) via passive diffusion from the suspension medium, i.e., solution A. Afterwards, 5 \(\mu\)l of the vesicle suspension was placed on the side of a polystyrene Eppendorf tube containing 95 \(\mu\)l of K-reaction medium: 160 mM KCl, 0.1 mM CaCl\(_2\), 10 \(\mu\)Ci 45CaCl\(_2\) 0.2 mM EGTA, 2 \(\mu\)M valinomycin, and 20 mM HEPES-Tris (pH 7.4). The free Ca\(^{2+}\) concentration in the medium was 50 mM as derived from calculation with the computer program EqCal for Windows (Bissof, 1996) for Ca-EGTA buffer. The Ca\(^{2+}\) influx was stopped by diluting the reaction mixture with 5 ml of ice-cold termination medium (160 mM KCl and 2 mM LaCl\(_3\)). Na\(^+-\)-dependent specific Ca\(^{2+}\) uptake was defined as the total Ca\(^{2+}\) uptake minus unspecific Ca\(^{2+}\) uptake in medium A containing 0.2 mM EGTA, 0.1 mM CaCl\(_2\), 10 \(\mu\)Ci 45CaCl\(_2\), and 2 \(\mu\)M valinomycin, i.e., in a solution where no Na\(^+\) gradient existed across the membrane. All samples were filtered under vacuum, and filters (GF/F, Whatman) were washed twice with 6 ml of 140 mM KCl-0.1 mM LaCl\(_3\). The protein content of each sample was determined with a kit from Bio-Rad with BSA as a standard.

Western blotting for SERCA2 and RyR. Membrane proteins from the right ventricular myocardium of each heart were extracted as described previously (39). Briefly, the right ventricular tissue was homogenized with a homogenizer (Polytron, PT 35 type) at 100 mg of tissue/ml ice-cold STE buffer (consisted of sucrose, Tris, EGTA, NaN\(_3\), NAF, PMSF, pepstatin A, leupeptin, and β-mercaptoethanol) and centrifuged at 1,000 \(\times\) g for 10 min. The supernatant was then centrifuged at 100,000 \(\times\) g for 60 min at 4\(^\circ\)C. The pellet from 100,000 \(\times\) g centrifugation was the cellular membrane fraction. It was previously shown that the membrane fraction did not...
contain any detectable collagens and was suitable for immunoblotting of SERCA and RyR (1). The protein concentration of the samples was quantified by the Bio-Rad protein assay method (6), using BSA for the standard curve.

For expression of SERCA2/RyR, 60 μg/30 μg of protein was blotted on one lane of a 12%/8% SDS-PAGE, and transferred electrophoretically to polyvinylidene difluoride membranes (0.2-μm pore size; Bio-Rad) at 4°C in a transfer buffer consisting of glycine and Tris) containing 20% methanol with the Bio-Rad Trans-blot electrophoretic transfer system. For RyR protein, which has a greater molecular weight than SERCA2, a special transfer solution (containing SDS in addition to glycine, Tris, and methanol) with a longer transferring time was used. After blocking with Tris-buffered saline (TBS; composed of Tris, NaCl, and 0.2% Tween 20) containing 0.5% nonfat milk, the membranes were incubated overnight at 4°C with the anti-SERCA2 antibody at 1:400 or the anti-ryanodine antibody at 1:1,800. The second antibody for both protein determinations was anti-goat antibody conjugated to horseradish peroxidase at 1:2,000 in 5% nonfat milk-TBS-Tween 20 for 1 h at room temperature. The proteins of SERCA2 and RyR were detected by the chemiluminescence method (ECL Western blotting detection; Amersham Biosciences). After immunoblotting, the film was scanned (HP Scanjet XPA 7400C) and the intensity of the bands was calculated with image analysis software (Quantity One, version 4.2.2; Bio-Rad). The density of proteins from the hypoxic rat and its corresponding age-matched normoxic control rat run in the same gel were compared, and the level of each protein sample in the hypoxic rat heart was expressed as a percentage of that in its age-matched control sample in the same gel. This analysis procedure can effectively eliminate not only the possible false results due to errors during protein loading, separation, and transfer but also the variations among experiments in protein density determinations of the bands (8, 25, 41).

Drugs and chemicals. Fura 2-AM, type I collagenase, ATP disodium salt, LaCl₃, isoproterenol, propranolol, valinomycin, EGTA, NaN₃, NAF, PMSF, pepstatin A, leupeptin, and β-mercaptoethanol were purchased from Sigma. Ryanodine, CPA, Ru-360, and calmidazolium were obtained from Calbiochem, and 45CaCl₂ was purchased from Amersham. All of the chemicals except fura 2-AM and CPA, which was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 0.1%, composed of Tris, NaCl, and 0.2% Tween 20) containing 0.5% nonfat milk-TBS-Tween 20 for 1 h at room temperature. The proteins of SERCA2 and RyR were detected by the chemiluminescence method (ECL Western blotting detection; Amersham Biosciences). After immunoblotting, the film was scanned (HP Scanjet XPA 7400C) and the intensity of the bands was calculated with image analysis software (Quantity One, version 4.2.2; Bio-Rad). The density of proteins from the hypoxic rat and its corresponding age-matched normoxic control rat run in the same gel were compared, and the level of each protein sample in the hypoxic rat heart was expressed as a percentage of that in its age-matched control sample in the same gel. This analysis procedure can effectively eliminate not only the possible false results due to errors during protein loading, separation, and transfer but also the variations among experiments in protein density determinations of the bands (8, 25, 41).

RESULTS

Time course of changes in electrically induced [Ca²⁺]ᵢ transient in right ventricular myocytes of normoxic and CH rats. The amplitude of the electrically induced [Ca²⁺]ᵢ transient was not altered in CH rats treated for 1, 3, or 7 days compared with those in the age-matched controls. However, after hypoxia for 2 wk, the amplitude of the [Ca²⁺]ᵢ transient decreased significantly (Fig. 2, A and B). The decline was aggravated in the 21- and 28-day CH groups, and there was no further decrease in the amplitude after CH treatment for 2 mo (Fig. 2B). Levels of diastolic [Ca²⁺]ᵢ in the myocytes
were not different between the CH and normoxic groups at any time points (Table 2).

As shown in Fig. 2, starting at 2 wk of CH, the time from resting to peak of electrically induced [Ca\(^{2+}\)], transient, which represents the speed of release of Ca\(^{2+}\) from SR, was prolonged and the value was significantly increased by 21 days. The time to peak value further increased in the 28-day CH group and remained at this level in the 56-day group (Fig. 2, A and C).

The \(\tau\) value, which represents the decline from peak of the electrically-induced [Ca\(^{2+}\)], transient and is an indicator of uptake of Ca\(^{2+}\) by Ca\(^{2+}\) ATPase, was not different from that in the normoxic controls in the first 2 wk of CH treatment (Fig. 2D). However, similar to the temporal changes of the time to peak values, the \(\tau\) value was significantly increased in the 3-wk and 1- and 2-mo CH groups relative to age-matched normoxic control animals. There was no difference between the \(\tau\) values of the transient after CH for 1 and 2 mo.

**Time course of changes in caffeine-induced [Ca\(^{2+}\)], transient in right ventricular myocytes of normoxic and CH rats.** The amplitude of the caffeine-induced [Ca\(^{2+}\)], transient, which reflects the content of Ca\(^{2+}\) in SR as caffeine depletes it, remained at levels comparable to those in the controls during the first week of CH treatment but gradually decreased from the 14th day of hypoxia. The amplitude was significant attenuated in the 21- and 28-day CH groups compared with the normoxic control animals (Fig. 3, A and B). There was no significant difference between the 28- and 56-day groups (Fig. 3B). In addition, the falling phase of the caffeine-induced [Ca\(^{2+}\)], transient, an indication of NCX activity, was also prolonged (Fig. 3, A and C). The changes in \(\tau\) values of the transient after CH paralleled the changes in amplitude (Fig. 3, B and C).

**Time course of changes in function and expression of SERCA2 and RyR proteins of SR in right ventricular myocytes of normoxic and CH rats.** To study SR function, we first determined the \(^{45}\)Ca\(^{2+}\) uptake via SERCA by SR. The uptake was significantly reduced in rats subjected to hypoxia for 2 wk and was further reduced after 4 and 8 wk of hypoxia (Fig. 4). The maximum reduction was 70% of the corresponding control animal.

The expression of SERCA2 protein showed similar time course changes except that in the heart of rats subjected to CH for 2 wk, the expression of SERCA2 seemed to be reduced; the difference was, however, not statistically significant (Fig. 5).

Ryanodine-sensitive \(^{45}\)Ca\(^{2+}\) uptake, which was an indication of Ca\(^{2+}\) release via the RyR, also displayed a significant reduction starting after 2 wk of hypoxia (Fig. 6). Further reductions occurred with longer durations of hypoxia. The maximum reduction occurred after 28 days of hypoxia, when it reached 120% of the corresponding normoxic control animal. In contrast to the ryanodine-sensitive \(^{45}\)Ca\(^{2+}\) uptake, the expression of RyR was not altered by hypoxia for up to 56 days (Fig. 7).

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**Table 2. Effects of chronic hypoxia on diastolic [Ca\(^{2+}\)], in rat right ventricular myocytes**

<table>
<thead>
<tr>
<th>Groups, days</th>
<th>Normoxic</th>
<th>Hypoxic</th>
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<tbody>
<tr>
<td>1</td>
<td>0.73 ± 0.04</td>
<td>0.73 ± 0.03</td>
</tr>
<tr>
<td>3</td>
<td>0.71 ± 0.05</td>
<td>0.72 ± 0.03</td>
</tr>
<tr>
<td>7</td>
<td>0.72 ± 0.03</td>
<td>0.72 ± 0.04</td>
</tr>
<tr>
<td>14</td>
<td>0.74 ± 0.02</td>
<td>0.75 ± 0.03</td>
</tr>
<tr>
<td>21</td>
<td>0.73 ± 0.04</td>
<td>0.73 ± 0.05</td>
</tr>
<tr>
<td>28</td>
<td>0.75 ± 0.05</td>
<td>0.75 ± 0.05</td>
</tr>
<tr>
<td>56</td>
<td>0.75 ± 0.04</td>
<td>0.77 ± 0.05</td>
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</tbody>
</table>

Values (F\(_{340}/F_{380}\)) are means ± SE; n = 10 rats in each group. [Ca\(^{2+}\)], intracellular Ca\(^{2+}\) concentration.
Time course of changes in NCX activity in right ventricular myocytes of normoxic and CH rats. NCX activity was significantly reduced in rats subjected to hypoxia for as long as a week and was further reduced with longer exposure (Fig. 8). A maximum reduction of 40% and plateau were reached after 4 wk of hypoxia.

Time course of changes in electrically induced [Ca2+]i transient in response to β-AR stimulation in right ventricular myocytes of normoxic and CH rats. To correlate the Ca2+ responses to β-AR stimulation with the altered Ca2+ homeostasis, we determined the time-dependent changes in the electrically induced [Ca2+]i transient in response to β-AR stimulation in right ventricular myocytes of normoxic and CH rats. A previous study in our laboratory (26) showed that isoproterenol at 0.01–10 μM increased the amplitude of the electrically induced [Ca2+]i transient in rat myocytes dose-dependently, with a plateau at 1 μM, and the effects of 1 μM isoproterenol were blocked by propranolol, a β-AR antagonist. In this study, we chose the concentration of 1 μM. The responses to 1 μM isoproterenol of the 1-, 3-, and 7-day CH groups were similar to those of the normoxic groups (Fig. 9). However, the response was significantly attenuated by 2 wk of CH treatment and further reduced after 21 and 28 days (Fig. 9). There was no significant difference between the 28- and 56-day groups.

DISCUSSION

In previous studies (26, 27), we showed that after 4 wk of hypoxia the electrically induced [Ca2+]i transient, which had been shown to directly correlate with shortening in the cardiomyocyte (40), was reduced. This observation suggests that Ca2+ homeostasis may be impaired after CH. In the present study, we attempted to characterize Ca2+ handling in the cardiomyocyte of CH rats. In addition to the electrically induced [Ca2+]i transient, we determined the time-dependent changes of the caffeine-induced [Ca2+]i transient, which indicates the Ca2+ content of the SR. We found that, like the change in the electrically induced [Ca2+]i transient, the amplitude of the caffeine-induced [Ca2+]i transient was also significantly re-
duced after 4 wk of hypoxia, indicating a reduction in the Ca\(^{2+}\) content of the SR after CH. Further analysis of the characteristics of the electrically induced [Ca\(^{2+}\)]\(_i\) transient showed that its time to peak and decay were significantly attenuated, suggesting reductions in release of Ca\(^{2+}\) from SR and reuptake of Ca\(^{2+}\) back to SR. The decay of the caffeine-induced [Ca\(^{2+}\)]\(_i\) transient was also attenuated, suggesting a reduction in NCX activity. To confirm these findings we measured the \(^{45}\text{Ca}^{2+}\) uptake via SERCA and release via RyR and the \(^{45}\text{Ca}^{2+}\) transport via NCX. We found that both the uptake of \(^{45}\text{Ca}^{2+}\) via SERCA and the release of \(^{45}\text{Ca}^{2+}\) via RyR were significantly suppressed in CH. NCX activity was also reduced. These observations were in good agreement with the findings on the Ca\(^{2+}\) transients. Therefore, in CH both the release and uptake of Ca\(^{2+}\) in SR and sarcolemmal NCX were suppressed, which may contribute to impaired Ca\(^{2+}\) handling, leading to reduced Ca\(^{2+}\) content in SR and a reduced electrically induced [Ca\(^{2+}\)]\(_i\) transient. It has been reported that Ca\(^{2+}\) influx via the L-type Ca\(^{2+}\) channel is significantly reduced and accompanied by a reduction in the electrically induced [Ca\(^{2+}\)]\(_i\) transient in the heart during CH (9), suggesting that a reduction in Ca\(^{2+}\) influx via the L-type Ca\(^{2+}\) channel may also contribute to the impaired Ca\(^{2+}\) handling in CH.

In the present study we found that CH attenuated the expression of the protein SERCA2, which correlated well with the depressed \(^{45}\text{Ca}^{2+}\) uptake via SERCA and the decay of the electrically induced [Ca\(^{2+}\)]\(_i\) transient. This observation suggests that the reduced expression of the protein is most likely responsible for the reduced Ca\(^{2+}\) uptake by SR. In contrast, the expression of RyR protein did not change. Therefore, the reduction in release of Ca\(^{2+}\) from SR is not caused by suppressed expression of the protein. We revealed that CH significantly attenuates the Ca\(^{2+}\) content in SR, as indicated by a reduction in the caffeine-induced [Ca\(^{2+}\)]\(_i\) transient. We suggest that the decrease in Ca\(^{2+}\) content of SR may be mainly respon-
sible for the reduction in Ca\(^{2+}\) release via RyR and is a result of suppressed expression of SERCA.

Because the \(\tau\) values of the electrically induced and caffeine-induced [Ca\(^{2+}\)] transient represent the decay of Ca\(^{2+}\) from the cytoplasm and extrusion of Ca\(^{2+}\) via the NCX, the difference of the \(\tau\) values indicates the Ca\(^{2+}\) uptake via the SERCA. With these values we could calculate the relative contributions of the SERCA and NCX to the decay or removal of Ca\(^{2+}\) after contraction. After 4 wk of hypoxia, the relative contributions of SERCA and NCX to the removal of Ca\(^{2+}\) were 82% and 18%, respectively, in the heart of hypoxic rats, which were similar to the corresponding values of 80% and 20%, respectively, in the normoxic group. On the basis of \(45\) Ca\(^{2+}\) experiments, the relative contributions of SERCA and NCX were 95% and 5%, respectively, in both normoxic and hypoxic groups. The finding indicates that the SERCA and NCX activities were suppressed in hypoxia to a similar extent.

One of the important findings in this study was that impaired Ca\(^{2+}\) handling in SR and sarcolemma occurred between 1 and 3 wk of hypoxia. Maximum changes occurred after 4 wk of CH. This finding is in agreement with previous observations that 4 wk is required for full adaptation to hypoxia (20, 21). Interestingly, the time course of the Ca\(^{2+}\) response to isoproterenol paralleled the time course of Ca\(^{2+}\) handling, suggesting that the attenuated Ca\(^{2+}\) response to \(\beta\)-AR stimulation may be due, at least in part, to impaired Ca\(^{2+}\) handling during CH.

The reductions in the electrically induced [Ca\(^{2+}\)] transient and shortening of myocytes from both normoxic and CH rats have been shown to be directly correlated (26, 27). This observation supports the notion that the reduction in contractility in CH is secondary to the reduction in the electrically induced [Ca\(^{2+}\)] transient and suggests that it is unlikely that the reduction in contractility is due to a change in Ca\(^{2+}\)-myofilament affinity.

In conclusion, the present study has characterized for the first time the impaired handling of Ca\(^{2+}\) in SR and sarcolema of myocytes from rats subjected to CH. The impairment includes reduced expression of and Ca\(^{2+}\) uptake by SERCA, reduced release of Ca\(^{2+}\) via RyR, and reduced NCX activity. The impairment in turn leads to reductions in Ca\(^{2+}\) content in SR, in the electrically induced [Ca\(^{2+}\)] transient, and in contractility. The impaired Ca\(^{2+}\) handling may be responsible for the attenuated Ca\(^{2+}\) response to \(\beta\)-AR stimulation.

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DISCLOSURES

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REFERENCES


24. C1428 CALCIUM HOMEOSTASIS IN CHRONIC HYPOXIA


