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C. Wang, J.-F. Du, F. Wu and H.-C. Wang

Am J Physiol Heart Circ Physiol, June 1, 2008; 294 (6): H2540-H2546.

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Chronic intermittent hypoxia alters Ca^{2+} handling in rat cardiomyocytes by augmented $\text{Na}^+/\text{Ca}^{2+}$ exchange and ryanodine receptor activities in ischemia-reperfusion

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Yeung HM, Kravtsov GM, Ng KM, Wong TM, Fung ML. Chronic intermittent hypoxia alters Ca^{2+} handling in rat cardiomyocytes by augmented $\text{Na}^+/\text{Ca}^{2+}$ exchange and ryanodine receptor activities in ischemia-reperfusion. *Am J Physiol Cell Physiol* 292: C2046–C2056 2007. First published January 31, 2007; doi:10.1152/ajpcell.00458.2006.—This study examined Ca^{2+} handling mechanisms involved in cardioprotection induced by chronic intermittent hypoxia (CIH) against ischemia-reperfusion (I/R) injury. Adult male Sprague-Dawley rats were exposed to 10% inspired O_2 continuously for 6 h daily from 3, 7, and 14 days. In isolated perfused hearts subjected to I/R, CIH-induced cardioprotection was most significant in the 7-day group with less infarct size and lactate dehydrogenase release, compared with the normoxic group. The I/R-induced alterations in diastolic Ca^{2+} level, amplitude, time-to-peak, and the decay time of both electrically and caffeine-induced Ca^{2+} transients measured by spectrofluorometry in isolated ventricular myocytes of the 7-day CIH group were less than that of the normoxic group, suggesting an involvement of altered Ca^{2+} handling of the sarcoplasmic reticulum (SR) and sarcolemma. We further determined the protein expression and activity of $^{45}\text{Ca}^{2+}$ flux of SR- Ca^{2+} -ATPase, ryanodine receptor (RyR) and sarcolemmal $\text{Na}^+/\text{Ca}^{2+}$ exchange (NCX) in ventricular myocytes from the CIH and normoxic groups before and during I/R. There were no changes in expression levels of the Ca^{2+} -handling proteins but significant increases in the RyR and NCX activities were remarkable during I/R in the CIH but not the normoxic group. The augmented RyR and NCX activities were abolished, respectively, by PKA inhibitor (0.5 μM KTS720 or 0.5 μM PKI₁₄₋₂₂) and PKC inhibitor (5 μM chelerythrine chloride or 0.2 μM calphostin C) but not by Ca^{2+} /calmodulin-dependent protein kinase II inhibitor KN-93 (1 μM). Thus, CIH confers cardioprotection against I/R injury in rat cardiomyocytes by altered Ca^{2+} handling with augmented RyR and NCX activities via protein kinase activation.

cardioprotection; intracellular calcium

CALCIUM (Ca^{2+}) plays an important role in normal cardiac functions. The sarcoplasmic reticulum (SR) and sarcolemma of the cardiomyocyte are the major components of maintaining Ca^{2+} homeostasis in the heart (23). The depolarization of the action potential activates L-type Ca^{2+} channels and results in Ca^{2+} influx for an increase in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$). This small rise in $[\text{Ca}^{2+}]_i$ triggers the release of Ca^{2+} from the SR via ryanodine receptors (RyRs), by the process known as Ca^{2+} -induced Ca^{2+} release (5). During relaxation, most of the Ca^{2+} released is sequestered back to the SR via SR- Ca^{2+} -

ATPase (SERCA) and partly extruded out of the cell via the sarcolemmal $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX). It has been shown that ischemia-reperfusion (I/R) depresses SR functions by depressing phosphorylation of SR Ca^{2+} handling proteins (30) and results in impaired Ca^{2+} homeostasis leading to intracellular Ca^{2+} overload and decreasing myocardial contractility in rat cardiomyocytes (3, 10, 33). Thus activation of regulatory kinases of SR Ca^{2+} handling proteins may attenuate impairment of Ca^{2+} homeostasis in myocardial injury during I/R.

Hypoxia preconditioning with continuous hypoxia (CH) or intermittent high-altitude hypoxia (IAH) increases myocardial tolerance to I/R (52). However, acclimatization to CH is associated with cardiac hypertrophy subsequent to the responses to hypoxia with polycythemia, pulmonary vascular remodeling and hypertension. In addition, Ca^{2+} handling alters in CH cardiomyocytes. The reductions in SERCA2 expression and in activities of SERCA, RyR and NCX significantly contribute to the impaired Ca^{2+} homeostasis and responsiveness to β -adrenoceptor stimulation in CH cardiomyocytes (31, 32). These could have deleterious effects on the myocardial functions leading to heart failure in pathophysiological conditions (12, 14). In contrast, despite the chronically hypobaric exposure for 42 days, IAH confers cardioprotection with negligible impacts reported on the heart and the Ca^{2+} handling in the cardiomyocyte (7, 8, 26, 27). Moreover, it has also been shown that chronic intermittent hypoxia (CIH) for short term in normobaric conditions increases tolerance of rat hearts against ischemic injury (2).

Cellular mechanisms involved in IAH-induced cardioprotection have been shown including activation of ATP-sensitive potassium channel, increase in endogenous nitric oxide production and upregulation of protein kinase C activity (27, 40, 42, 50, 51). More recently, alterations in Ca^{2+} handling have also been indicated in IAH-induced cardioprotection against I/R injury with changes in SR function, NCX activity, and phosphorylation of phospholamban (6, 47). Given that phosphorylation of SR and sarcolemmal Ca^{2+} handling proteins attenuates the dysfunctions of SR and sarcolemma during I/R (29), we hypothesized that alterations in the activity and expression of Ca^{2+} handling proteins of SR and sarcolemma account for the ameliorated Ca^{2+} homeostasis in CIH-induced cardioprotection against I/R injury. To test the hypothesis, we first determined the I/R injury in the heart of CIH rats and the

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Ca^{2+} handling in CIH ventricular myocytes subjected to metabolic inhibition/anoxia and reperfusion (MI/A-R). We further examined the rate of $^{45}\text{Ca}^{2+}$ uptake by SERCA, release by RyR and extrusion by sarcolemmal NCX and their protein expressions. Moreover, we examined the involvement of activation of regulatory kinases of SR Ca^{2+} handling proteins with protein kinase A (PKA), protein kinase C (PKC), and Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) in the altered Ca^{2+} handling of SR and sarcolemma in CIH ventricular myocytes during MI/A-R.

MATERIALS AND METHODS

The protocols of this study were approved by the Committee on the Use of Experimental Animals for Teaching and Research, The University of Hong Kong. Animals were maintained in our animal facilities on standard laboratory chow and received care in compliance with the requirements of the University of Hong Kong and the National Institutes of Health guidelines.

Animal preparations. Adult male Sprague-Dawley rats weighed 290–320 g at the start of the experiment were randomly divided into two groups. One group was treated with CIH and the normoxic control was maintained in animal cages in room air. For CIH, rats were exposed to inspired oxygen, $\sim 10\%$ O_2 for 6 h per day in an acrylic chamber. The hypoxic environment was maintained with inflow of mixture of room air and nitrogen that was regulated by an oxygen analyzer (model 1755518A, Gold Edition, Vacuum Med). The animals had free access to the food and water. Rats were exposed to CIH for 3, 7, and 14 days. The CIH protocol is generally relevant to sojourns to high altitude and intermittent hypoxic training, which were reported to induce cardioprotection against ischemic insults (8, 27). Experiments were performed immediately after removal of the rats from the chamber. The rats were decapitated and the hearts were quickly removed. The hematocrit index, body and heart weights from all rats were recorded.

Isolated perfused heart preparation. Rats from normoxic and CIH groups (3, 7, and 14 days, respectively) were decapitated and the hearts were quickly removed and placed in ice-cold Krebs-Henseleit (K-H) perfusion buffer before being mounted on the Langendorff apparatus for perfusion at 37°C with K-H buffer at constant pressure (100 cm of H_2O) and equilibrated with 95% O_2 - 5% CO_2 . The buffer contained (in mM) 118.0 NaCl, 4.7 KCl, 1.25 CaCl_2 , 1.2 KH_2PO_4 , 1.2 MgSO_4 , 25.0 NaHCO_3 , and 11.0 glucose. All of the hearts were subjected to regional ischemia described previously (4). Briefly, a snare was formed by placing a silk suture around the left coronary artery of the rat heart. The snare was pulled to occlude the coronary artery to produce ischemia. Reperfusion was done by releasing the snare. In this study, the isolated rat hearts were subjected to 30 min of ischemia, followed by 120 min of reperfusion, which induced myocardial injury.

Measurement of the area of risk. To determine the infarct size, the coronary artery was reoccluded at the end of reperfusion and the heart was perfused with 2.5% Evans blue dye to delineate the area of risk. The hearts were frozen at -70°C , cut into thin slices, which were perpendicular to the septum, from the apex to the base. Then the slices were incubated in sodium phosphate buffer containing 1% (wt/vol) 2,3,5-triphenyl-tetrazolium chloride for 10 min to visualize the unstained infarct region. The infarct and the risk zone areas were determined by planimetry with Image J software from the National Institutes of Health (NIH). The infarct size measured was expressed as a percentage of the risk zone.

Determination of myocardial injury by lactate dehydrogenase efflux. The effluent from each isolated perfused rat heart was collected at exactly 5 min of reperfusion and the LDH was assayed spectrophotometrically by using a kit purchased from Sigma-Aldrich

(St. Louis, MO). The LDH activity measured was expressed as units per liter.

Preparation of isolated ventricular myocytes. Single ventricular myocytes were isolated from the normoxic and 7-day CIH rats by using a collagenase method described previously (46). After isolation, myocytes were allowed to stabilize for at least 30 min before any experiment. For ischemic insults, myocytes were incubated for 10 min with nonglucose K-H solution containing 10 mM 2-deoxy-D-glucose and 10 mM sodium dithionite to induce MI/A. Reperfusion was followed by incubating the myocytes with normal K-H solution for further 10 min.

Measurement of $[\text{Ca}^{2+}]_i$. A spectrofluorometric method with fura 2-AM as a Ca^{2+} indicator was used during the measurement of $[\text{Ca}^{2+}]_i$. Ventricular myocytes from either normoxic or 7-day CIH rat group were incubated with 5 μM fura 2-AM for 35 min. Fluorescent signals obtained at 340 nm (F_{340}) and 380 nm (F_{380}) excitation wave-lengths were recorded and stored in computer for data processing and analysis. The F_{340}/F_{380} ratio was used to indicate cytosolic $[\text{Ca}^{2+}]_i$ in the ventricular myocytes. During the measurement of electrically induced $[\text{Ca}^{2+}]_i$ ($E[\text{Ca}^{2+}]_i$) transients, myocytes were electrically stimulated at 0.2 Hz, whereas measurement of caffeine-induced $[\text{Ca}^{2+}]_i$ ($C[\text{Ca}^{2+}]_i$) transients were done by applying 10 mM caffeine directly to the ventricular myocytes. The amplitude of $E[\text{Ca}^{2+}]_i$ and $C[\text{Ca}^{2+}]_i$ transients were determined as the difference between the resting and the peak $[\text{Ca}^{2+}]_i$ levels; the time for 50% decay of the transients (T_{50}) was used to represent the decay of both transients.

Western blot analysis for SERCA, RyR, and NCX. Myocytes from normoxic and 7-day CIH rat groups after subjected to 10-min MI/A and 10-min reperfusion were harvested. To detect the expression of SERCA and RyR, SR vesicles were obtained by following procedures. Briefly, myocytes were sonicated on ice in the extraction medium containing (in mM) 15 Tris, 10 NaHCO_3 , 5 NaN_3 , 250 sucrose, and 1 EDTA (pH 7.3). The homogenate were centrifuged for 10 min at 1,000 g to remove cellular debris. The supernatant was further centrifuged for 35 min at 20,000 g. Then the pellet was re-suspended in a mixture of 0.6 M KCl and 0.03 M histidine (pH 7.0) and centrifuged for 35 min at 20,000 g. The final pellet was re-suspended in a mixture of 0.25 M sucrose and 0.01 M histidine (pH 7.3) and stored at -70°C . All solutions were contained three proteases inhibitors: 1 mg/ml leupeptin, 1 mg/ml aprotinin, and 1 mM PMSF. For the measurement of NCX, purification of plasma membrane vesicles were carried out as described above. The pellet, i.e., sarcolemma-enriched fraction was dissolved in the lysis buffer (0.6 M sucrose and 10 mM imidazole-HCl, pH 7.0) and stored at -70°C . The protein concentration of the samples was quantified by the Bio-Rad protein assay method by using bovine serum albumin for the standard curve. Sample proteins (60 $\mu\text{g}/\text{lane}$) were separated in SDS-polyacrylamide gel (12% for SERCA and NCX; 8% for RyR) and transferred electrophoretically to polyvinylidene difluoride membranes (0.2 μm pore size; Bio-Rad) at 4°C in transfer buffer with glycine, Tris, and 20% methanol with the Bio-Rad Trans-blot electrophoretic transfer system. After being blocked with Tris-buffered saline (with Tris, NaCl, and 0.2% Tween 20) containing 5% nonfat milk, the membranes were incubated overnight at 4°C with the goat anti-SERCA2 polyclonal antibody (1:400 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-RyR2 monoclonal antibody (1:3,330 dilution; Affinity BioReagent, Golden, CO) and mouse anti-NCX1 monoclonal antibody (1:500 dilution; Abcam, Cambridge, UK). The second antibody for both protein determinations was either anti-goat or anti-mouse antibody conjugated to horseradish peroxidase (1:2,000 dilution; Dako Cytomation) in 5% nonfat milk Tris-buffered saline for 1 h at room temperature. The protein bands of SERCA, RyR, and NCX were detected by the chemiluminescence method (ECL Western blot analysis detection; Amersham Biosciences).

Isolation of SR and measurement of $^{45}\text{Ca}^{2+}$ uptake. SR vesicles were obtained by a method described previously (31) with some

modifications. Briefly, freshly isolated cardiac myocytes from rats were destroyed by a low temperature (-75°C) and homogenized in extraction medium containing (in mM) 40 imidazole-HCl, 10 NaHCO₃, 5 NaN₃, 250 sucrose, and 1 EDTA (to $\sim 2^{\circ}\text{C}$; pH 7.0; 5 ml/g tissue), with a Polytron PT 35 homogenizer (Brinkmann, Westbury, NY) at setting 9 for 10 s each. 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 in Sorvall SM-24 rotor and the supernatant was discarded. The pellet was suspended in 8 ml of a mixture 0.6 mM KCl and 20 mM imidazole-HCl (pH 7.0) and centrifuged at 48,000 *g* for 60 min in Sorvall SM-24 rotor. The final pellet was rehomogenized in 1 ml 250 mM sucrose and 40 mM imidazole-HCl using a Potter-Elvehjem homogenizer with Teflon pestle and stored at -70°C . All solutions contained three protease inhibitors: soybean trypsin inhibitor (40 $\mu\text{g}/\text{ml}$), 0.1% PMSF, and leupeptin (0.5 $\mu\text{g}/\text{ml}$).

The ATP-dependent transport Ca²⁺ to SR was measured at room temperature (22°C) using our method described earlier (19). SR protein (50–100 μg) 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 MgCl₂, 4 mM ATP-Na₂, 5 K-oxalate, 3 μCi ⁴⁵CaCl₂, 5 μM Ru360, an inhibitor of Ca²⁺ uptake in mitochondria (48), and 5 μM calmidazolium, an inhibitor of Ca²⁺-ATPase of the sarcolemma (25). A free [Ca²⁺] in this solution (5 μM) was determined by a Ca²⁺-EGTA buffer and calculated according to Fabiato and Fabiato (9). After 10 min, aliquots of 0.9 ml were filtered through filters (0.45 μm ; Millipore, Bedford, MA). Filters were washed three times with 4 ml of cold ($2^{\circ}\text{--}4^{\circ}\text{C}$) solution containing 40 mM imidazole-HCl (pH 7.0), 100 mM KCl, and 0.1 mM EGTA. After being washed, the Millipore filters were placed into vials containing 10 ml of scintillation cocktail (Universal LSC cocktail for aqueous samples, Sigma) and left for about 40 min. The radioactivity was then counted in scintillation counter (LS 6500, Beckman).

The ⁴⁵Ca²⁺ uptake by SERCA, representing the activity of SERCA, was defined as the difference between the rate of ⁴⁵Ca²⁺ uptake a K-oxalate containing solution in the presence and absence of 10 μM cyclopiazonic acid, a specific inhibitor of SERCA (37). The difference in uptake in the presence and absence of 50 μM ryanodine, a specific blocker of RyR, was defined as the ⁴⁵Ca²⁺ release via the RyR receptor.

Plasma membrane purification and NCX assay. For purification of plasma membrane vesicles the procedure described previously (31) was used with some modifications. Briefly, freshly isolated cardiac myocytes from rats were destroyed by low temperature (-75°C) 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 maximum speed with Polytron PT 35. The homogenate was centrifuged at 1,000 *g* for 5 min. The supernatant was centrifuged at 12,000 *g* for 10 min in Sorvall SM-24 rotor. The 12,000 *g* supernatant was diluted in 1.5 volumes of 160 mM NaCl and 20 mM HEPES-Tris (pH 7.4). This vesicle suspension was completed to 30 ml with the same solution supplemented with 0.25 M sucrose. The fraction was then centrifuged 160,000 *g* for 70 min (Beckman, L8-M; rotor Ti 50.4). The pellet representing the sarcolemma-enriched fraction was dissolved in 0.5 ml of *solution A*, which was composed of (in

mM) 100 NaCl, 50 LiCl, 6 KCl, 20 HEPES-Tris (pH 7.4), and assayed for Na⁺/Ca²⁺ exchange activity. All solutions contained all three protease inhibitors, 40 mg/ml soybean trypsin inhibitor, 0.1% PMSF, and 0.5 $\mu\text{g}/\text{ml}$ leupeptin.

Na⁺/Ca²⁺ exchange was estimated as a specific Na⁺-dependent Ca²⁺ uptake following the protocol described previously (11) with some modifications. Briefly, 4 μl of the vesicle suspensions were incubated for 50 min at 22°C to load by Na⁺ via passive diffusion with their suspension medium, i.e., *solution A*. Afterward, 15 μl of the vesicle suspension were placed on the side of polystyrene. Eppendorf tube containing 85 μl K-reaction medium: 160 mM KCl, 0.1 mM CaCl₂, 100 μCi ⁴⁵CaCl₂, 0.2 mM EGTA, 2 μM valinomycin, 2 μM Ru360 to prevent the contribution of Na⁺/Ca²⁺ exchange of mitochondria, and 20 mM HEPES-Tris (pH 7.4). The free [Ca²⁺] in medium was 55 μM as derived from the calculation using the computer program EqCal for Windows (Biosoft, 1996) for Ca²⁺-EGTA buffer. The Ca²⁺ influx was stopped by diluting the reaction mixture after 2, 5, or 10 s with 5 ml of ice-cold termination medium: 160 mM KCl and 2 mM LaCl₃. Na⁺-dependent specific Ca²⁺ uptake was defined as the total Ca²⁺ uptake minus unspecific Ca²⁺ uptake in *medium A* containing 0.2 mM EGTA, 0.1 mM CaCl₂, 100 μCi ⁴⁵CaCl₂, 2 μM valinomycin, i.e., in solution where no Na⁺ gradient existed across the membrane. All samples were filtered under through Millipore filters (0.45 μm) washed twice with 6 ml of 140 mM KCl and 0.1 mM LaCl₂. The radioactivity was then counted as described above. The protein content of each sample was determined by using the kit from Bio-Rad with bovine serum albumin as a standard.

Drugs and chemicals. Collagenase (type I), 2-deoxy-D-glucose, NaN₃, sodium dithionite, fura 2-AM, 2,3,5-triphenyltetrazolium chloride, EGTA, valinomycin, LaCl₃, leupeptin, and PMSF were purchased from Sigma-Aldrich (St. Louis, MO). Ru360, calmidazolium, cyclopiazonic acid, ryanodine, KT5720, calphostin C, PKA inhibitor 14-22 amide, cell permeable (PKI₁₄₋₂₂), KN-93, and chelerythrine chloride were purchased from Calbiochem. ⁴⁵CaCl₂ was purchased from Amersham.

Statistical analysis. Values are expressed as means \pm SE. The unpaired Student's *t*-test was used to determine the differences between two groups. One-way ANOVA (Dunnett's multiple-comparison test) was used to determine the differences among the multiple groups. The significance level was set at $P < 0.05$.

RESULTS

Effects of CIH on heart weight, body weight, and hematocrit index in rats. As shown in Table 1, there were no differences in the body weight and heart weight of rats between the CIH and age-matched normoxic groups, except that heart weight-to-body weight ratio was increased by 7.7% in the 14-day CIH group. The hematocrit, which reflects the proportion by volume of the blood that consists of red blood cells, was not different in the 3-day and 7-day CIH groups but the level was markedly elevated by 16.8% in the 14-day CIH group (Table 1).

Table 1. *Effects of CIH on body weight, heart weight, and hematocrit index in rats*

Groups, Days	Normoxia				Chronic Intermittent Hypoxia			
	BW, g	HW, mg	HW/BW, mg/g	Hct, %	BW, g	HW, mg	HW/BW, mg/g	Hct, %
3	334.3 \pm 3.8	1,476.0 \pm 19.1	4.4 \pm 0.08	46.0 \pm 0.7	329.8 \pm 3.2	1,420.8 \pm 28.4	4.4 \pm 0.08	46.0 \pm 1.1
7	324.5 \pm 3.9	1,456.0 \pm 23.4	4.5 \pm 0.08	46.7 \pm 0.9	327.8 \pm 4.0	1,472.3 \pm 22.5	4.5 \pm 0.08	47.2 \pm 0.7
14	406.5 \pm 5.7	1,600.8 \pm 15.4	3.9 \pm 0.04	50.0 \pm 1.0	364.8 \pm 7.1*	1,566.3 \pm 16.3	4.2 \pm 0.04†	58.4 \pm 1.7*

Values are means \pm SE; $n = 6$ rats per group. CIH, chronic intermittent hypoxia; BW, body weight; HW, heart weight; Hct, hematocrit index. * $P < 0.01$ and † $P < 0.001$ vs. corresponding normoxic group.

Effects of CIH on I/R injury in isolated perfused rat hearts. As shown in Fig. 1, the infarct size and LDH release of rat hearts subjected to I/R were not different between the 3-day CIH and its corresponding normoxic groups. The infarct size and LDH release were significantly reduced by 24.8% and 27.4%, respectively, in the 7-day CIH group and were by 16.3% and 10.7% in the 14-day CIH group, compared with its corresponding normoxic group.

Effects of CIH on diastolic $[\text{Ca}^{2+}]_i$ level, electrically induced $[\text{Ca}^{2+}]_i$ transient and caffeine induced $[\text{Ca}^{2+}]_i$ transient in rat ventricular myocytes subjected to MI/A reperfusion. To test the hypothesis that the Ca^{2+} handling was ameliorated in the CIH cardiomyocytes during I/R, we determined the dia-

stolic $[\text{Ca}^{2+}]_i$ level (Fig. 2), the characteristics of the electrically induced $[\text{Ca}^{2+}]_i$ transient ($E[\text{Ca}^{2+}]_i$) (Fig. 3) and caffeine-induced $[\text{Ca}^{2+}]_i$ transient ($C[\text{Ca}^{2+}]_i$) (Fig. 4) from the normoxic and 7-day CIH groups of ventricular myocytes subjected to 10-min MI/A and 10-min reperfusion, since the 7-day CIH hearts were most protected from I/R injury.

The fluorescence ratio (F_{340}/F_{380}), which indicates the cytosolic Ca^{2+} level (13, 21), was not different between the CIH and normoxic groups before MI/A. The ratio was markedly increased by 4.2% at the end of MI/A in the normoxic group (Fig. 2) but the increase was less in the CIH group at 3.4% of the value before MI/A. During 2–3 min of reperfusion, which is the peak value of $[\text{Ca}^{2+}]_i$ after MI/A (38), the fluorescence ratio was still markedly increased by 4.2% but the increase was significantly lower in the CIH group at 1.7% of the value before MI/A.

The amplitude of $E[\text{Ca}^{2+}]_i$, which reflects the Ca^{2+} release during excitation-contraction coupling and directly correlates with shortening in rat cardiomyocytes (32, 49), was not different between the CIH and normoxic groups before MI/A. The amplitudes were significantly decreased by MI/A-R (Fig. 3A) and the decrease in the CIH group was less than that of the normoxic group (58% vs. 67% of the level before MI/A, respectively).

The decay time (T_{50}) of $E[\text{Ca}^{2+}]_i$, which reflects mainly the Ca^{2+} reuptake to SR via SERCA (>90% Ca^{2+} in cytoplasm) and partly the extrusion to extracellular space by NCX (1, 22), was longer in the CIH than that of the normoxic group before MI/A and it was markedly prolonged during MI/A-R in both groups (Fig. 3B). The increase in the T_{50} value was significantly less in the CIH than that of the normoxic group, at 63.6% vs. 136.8% of the level before MI/A.

The time to peak of $E[\text{Ca}^{2+}]_i$, which indicates the speed of Ca^{2+} release via RyR of SR (31), was not different between the CIH and normoxic groups before MI/A. The value was markedly increased by 41.6% during MI/A-R in the normoxic group but the increase was significantly less in the CIH group at 9.2% of the value before MI/A (Fig. 3C).

The amplitude of caffeine induced $[\text{Ca}^{2+}]_i$ ($C[\text{Ca}^{2+}]_i$) which is an index of Ca^{2+} content in SR (13, 44), was lower in the CIH group but not significantly different from the normoxic group before MI/A. The amplitudes were decreased by MI/A-R (Fig. 4A) and the decrease was significant in the normoxic group but not in the CIH group, respectively by 31% vs. 11% of the level before MI/A.

The T_{50} of $C[\text{Ca}^{2+}]_i$, which mainly reflects sarcolemmal NCX activity during caffeine-induced RyR release of Ca^{2+} from SR (34, 44), was not different between the CIH and normoxic groups before MI/A. The T_{50} value was significantly increased after MI/A and R in the normoxic group by 62.7% of the value before MI/A (Fig. 4B) but the increase was significantly less in the CIH group at 15.5%.

Effects of CIH on functions and protein expressions of SERCA, RyR of SR and sarcolemmal NCX in rat ventricular myocytes subjected to MI/A-R. To address the hypothesis that alterations in the activity and expression of Ca^{2+} handling proteins of SR and sarcolemma account for the ameliorated Ca^{2+} homeostasis in CIH cardiomyocytes during MI/A-R, we examined the levels of activity (Fig. 5, A–C) and protein

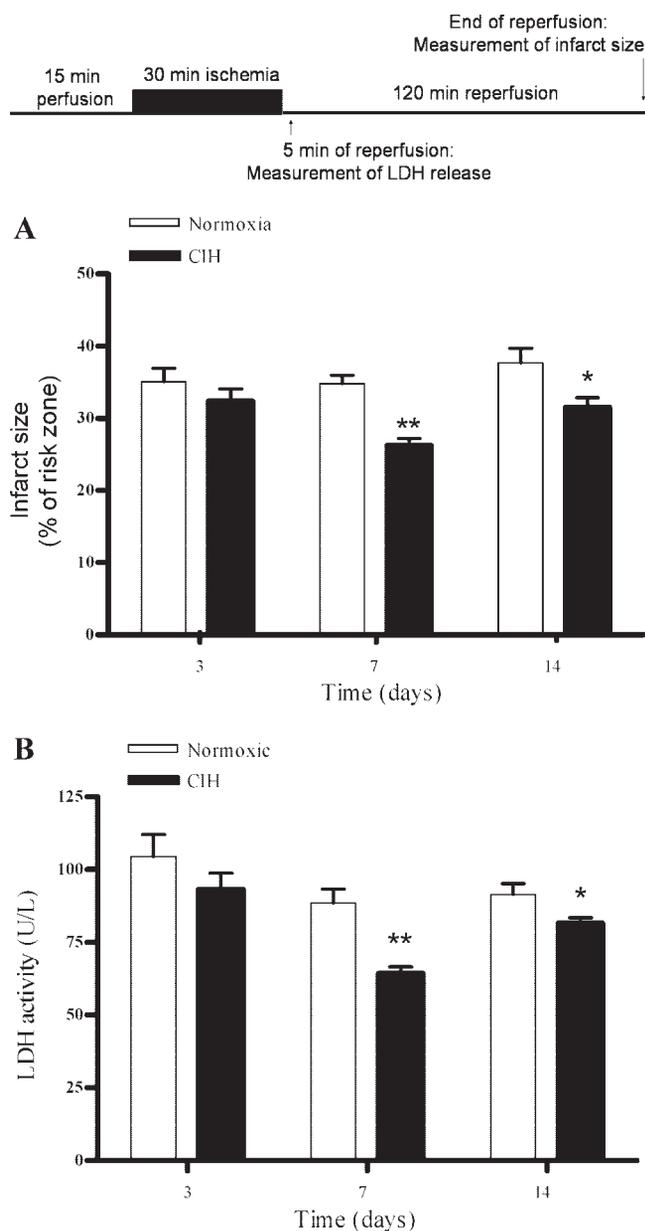


Fig. 1. Effects of chronic intermittent hypoxia (CIH) on myocardial infarct (A) and lactate dehydrogenase (LDH) release (B) in the isolated perfused heart subjected to 30 min of ischemia, followed by 120 min of reperfusion (I/R). Experimental protocol was shown at top. Values are means \pm SE; $n = 6$ rats in each group. ** $P < 0.01$ and * $P < 0.05$ vs. corresponding normoxic group.

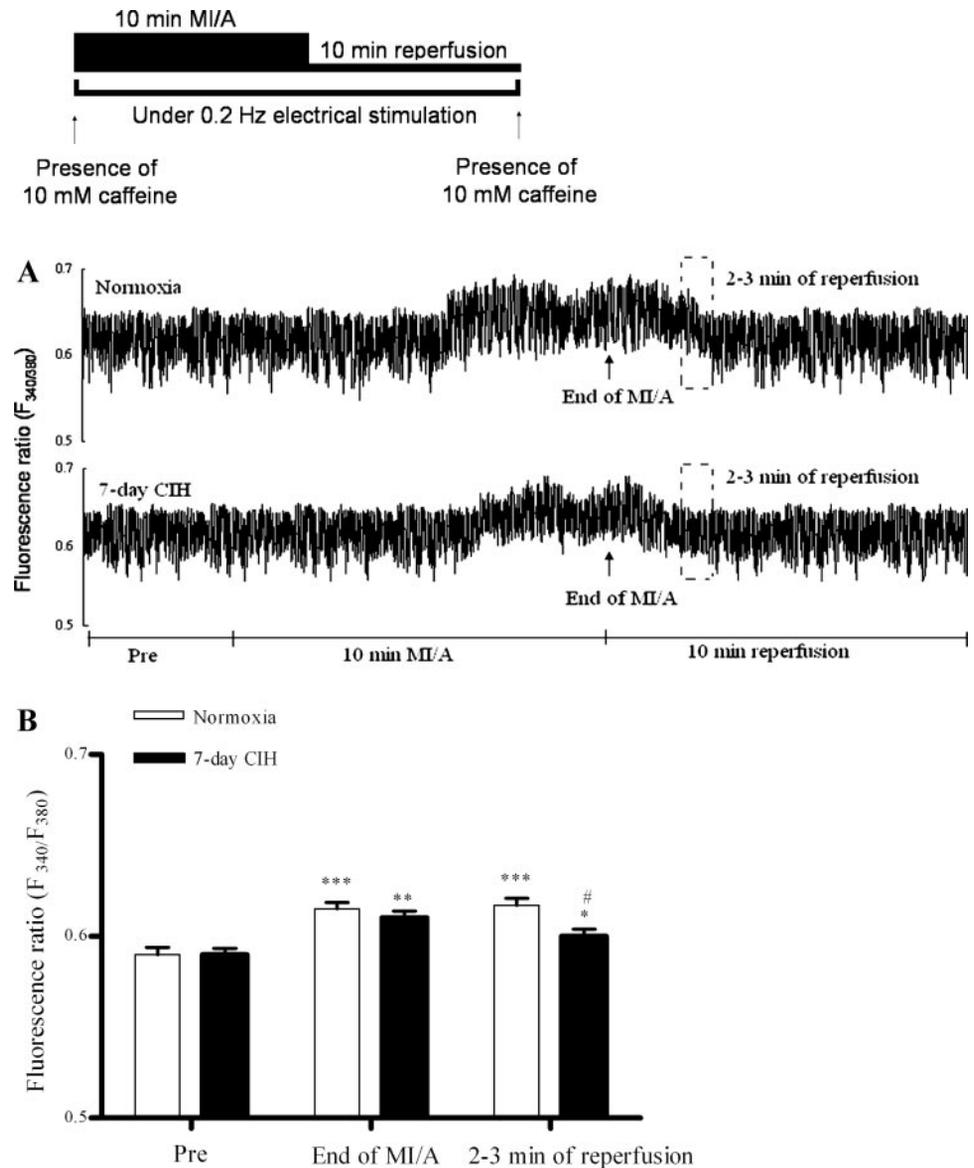


Fig. 2. Effects of CIH on fluorescence ratio of resting intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in ventricular myocytes subjected to metabolic inhibition/anoxia (MI/A) and reperfusion (R). Representative tracings recorded during the whole process are shown at top. Values are means \pm SE; $n = 4$ –5 myocytes from 4–6 rats in each group. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$ vs. corresponding pretreatment values (Pre) and # $P < 0.05$ vs. corresponding normoxic group.

expression (Figs. 6, A–C) of the SERCA, RyR, and NCX in the CIH and normoxic cardiomyocytes subjected to 10-min MI/A and 10-min R.

Before MI/A, the rate of the $^{45}\text{Ca}^{2+}$ uptake via SERCA by SR was significantly lower in the CIH compared with the normoxic group. Following MI/A-R, the uptake in the normoxic group was significantly reduced by 18.2% of the level before MI/A (Fig. 5A). In contrast, the rate in the CIH group was not significantly decreased by MI/A-R, and was not different from the normoxic group. The ryanodine-sensitive $^{45}\text{Ca}^{2+}$ uptake rate, which reflects the Ca^{2+} release via the RyR of SR, was not different between the CIH and normoxic groups before MI/A. The rate was not changed by MI/A-R in the normoxic group but was markedly increased in the CIH group by 30.2% of the level before MI/A (Fig. 5B). The rate of the extrusion of $^{45}\text{Ca}^{2+}$ by sarcolemmal NCX was not different between the CIH and normoxic groups before MI/A. Following MI/A-R, NCX activity in the normoxic group was significantly decreased by 19% of the level before MI/A, but was sharply increased by 43.6% in

the CIH group (Fig. 5C). There were no differences in the levels of protein expressions of SERCA2, RyR2, and NCX1 between the CIH and normoxic groups before MI/A (Fig. 6). Also, there were no changes in the expression levels of the proteins in the CIH and normoxic groups subjected to MI/A-R (Fig. 6).

Effects of blockade of PKA, PKC, and CaMKII on $^{45}\text{Ca}^{2+}$ uptake via SERCA, release via RyR of SR and extrusion by sarcolemmal NCX in CIH cardiomyocytes subjected to MI/A-R. Given the similar levels of protein expressions of SERCA2, RyR2, and NCX1, we further hypothesized a mechanistic role of phosphorylation mediated by regulatory kinases of the Ca^{2+} handling proteins in the augmented Ca^{2+} handling of SR and sarcolemma in the CIH cardiomyocytes. We determined the $^{45}\text{Ca}^{2+}$ uptake by SERCA, ryanodine-sensitive uptake by RyR and extrusion by sarcolemmal NCX in the CIH and normoxic groups subjected to 10-min MI/A and 10-min reperfusion, in the presence of PKA inhibitor (0.5 μM KT5720 or 0.5 μM myristoylated PKA inhibitor amide 14-22, cell-permeable, PKI₁₄₋₂₂), or

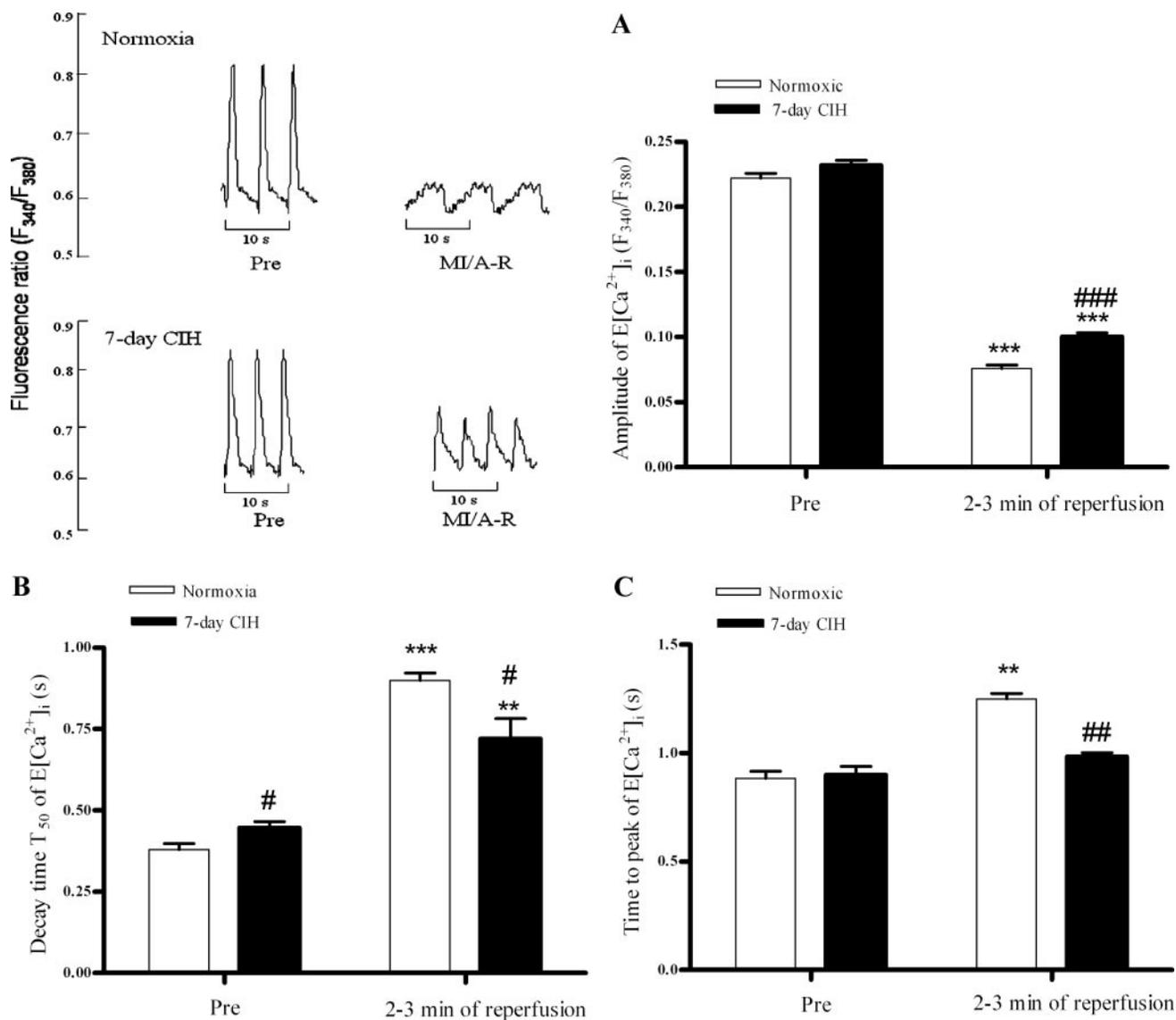


Fig. 3. Effects of CIH on amplitude (A), decay time (B), and time to peak (C) of electrically induced $[\text{Ca}^{2+}]_i$ transients in ventricular myocytes subjected to MI/A and R. Experimental protocol was same as in Fig. 2 and representative tracings recorded during the whole process are shown at top. Values are means \pm SE; $n = 4-6$ myocytes from 5-6 rats in each group. *** $P < 0.001$, ** $P < 0.01$ vs. corresponding pretreatment values (Pre). ### $P < 0.001$, ## $P < 0.01$ and # $P < 0.05$ vs. corresponding normoxic group.

PKC inhibitor (5 μM chelerythrine chloride or 0.2 μM calphostin C) or CaMKII inhibitor, 1 μM KN-93 (Fig. 7).

Results showed that the rate of the $^{45}\text{Ca}^{2+}$ uptake by SERCA remained unchanged in the CIH and normoxic groups with or without the presence of protein kinase inhibitors (Fig. 7, A and B). The rates of ryanodine-sensitive $^{45}\text{Ca}^{2+}$ release and $^{45}\text{Ca}^{2+}$ extrusion by sarcolemmal NCX were increased in the CIH compared with the normoxic group. The increased rate of ryanodine-sensitive $^{45}\text{Ca}^{2+}$ release was significantly blocked by PKA inhibitors but not by PKC or CaMKII inhibitors, and all inhibitors had no effects on the normoxic groups (Fig. 7, C and D). In contrast to RyR, the increased rate of $^{45}\text{Ca}^{2+}$ extrusion by sarcolemmal NCX was abolished by PKC inhibitors but not affected by PKA and CaMKII inhibitors. In addition, there was a lack of effect of all inhibitors on the normoxic groups (Fig. 7E and 7F).

DISCUSSION

Consistent with previous studies, our results showed that CIH confers cardioprotection against I/R injury (2, 8, 27). The finding that the cardioprotective mechanism was not significant in the heart of rats with 3-day CIH, but was most prominent in the 7-day group and gradually declined after CIH for 14 days, suggests that the cardioprotective mechanism is separate from the alterations following the acclimatization. In fact, the hematocrit index and the heart weight-to-body weight ratio were not changed in the 7-day but increased in the 14-day CIH group, indicating that the excessive erythrocytosis and cardiac changes during the acclimatization may have negative impacts on the cardiac functions in pathophysiological condition such as I/R (16, 18).

In addition, the Ca^{2+} kinetics were not significantly altered in the heart of rats with 7-day CIH, as we shown in the

$\text{E}[\text{Ca}^{2+}]_i$ and $\text{C}[\text{Ca}^{2+}]_i$ transients, and the activity and expression of the Ca^{2+} -handling proteins of the CIH cardiomyocytes before MI/A. Yet, the Ca^{2+} -handling mechanism was significantly changed by CIH resulting in attenuated intracellular

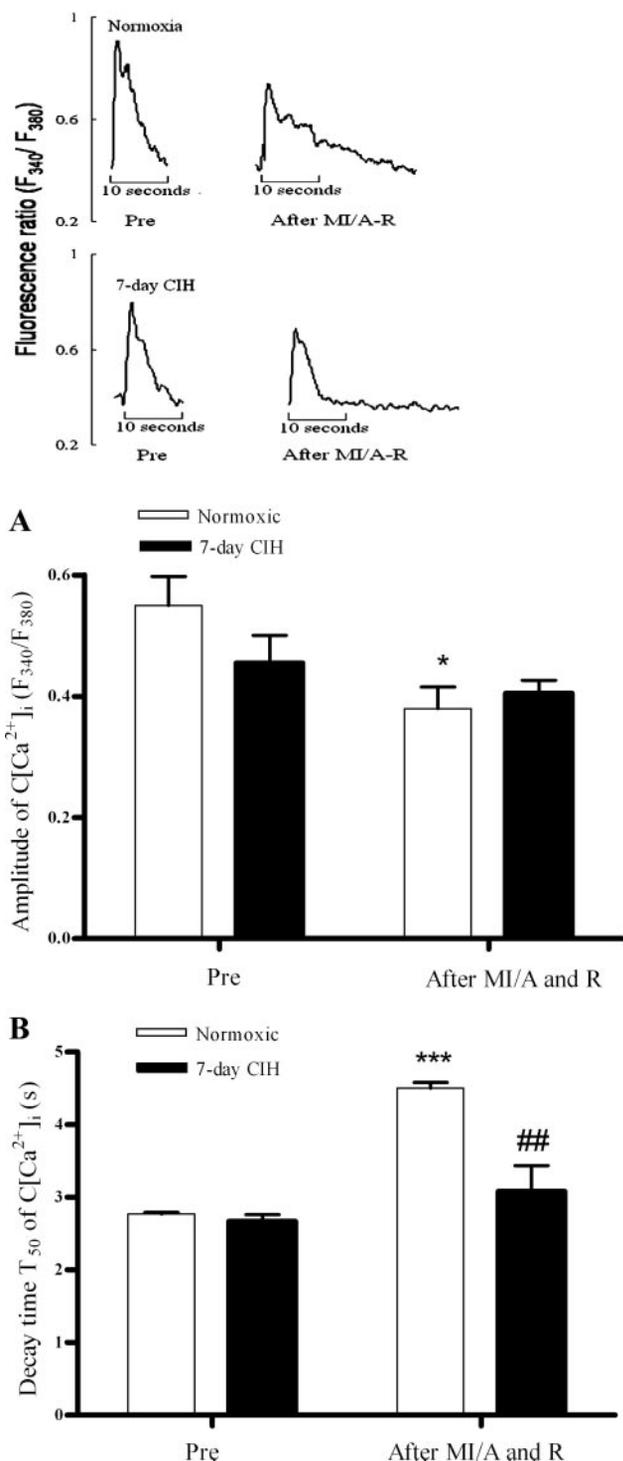


Fig. 4. Effects of CIH on amplitude (A) and decay time (B) of caffeine-induced $[\text{Ca}^{2+}]_i$ transients in ventricular myocytes subjected to MI/A and R. Experimental protocol was same as in Fig. 2 and representative tracings recorded during the whole process are shown at top. Values are means \pm SE; $n = 4-5$ myocytes from 4-6 rats in each group. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$ vs. corresponding pretreatment values (Pre); ## $P < 0.01$ and # $P < 0.05$ vs. corresponding normoxic group.

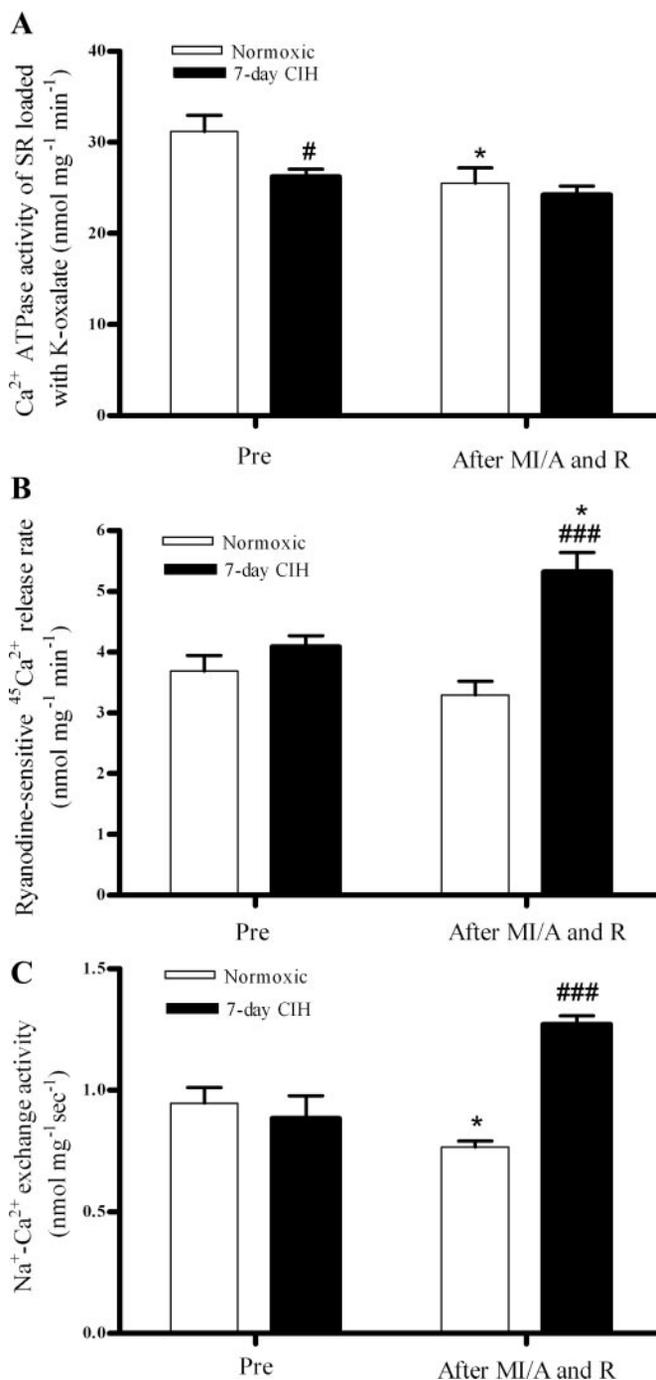
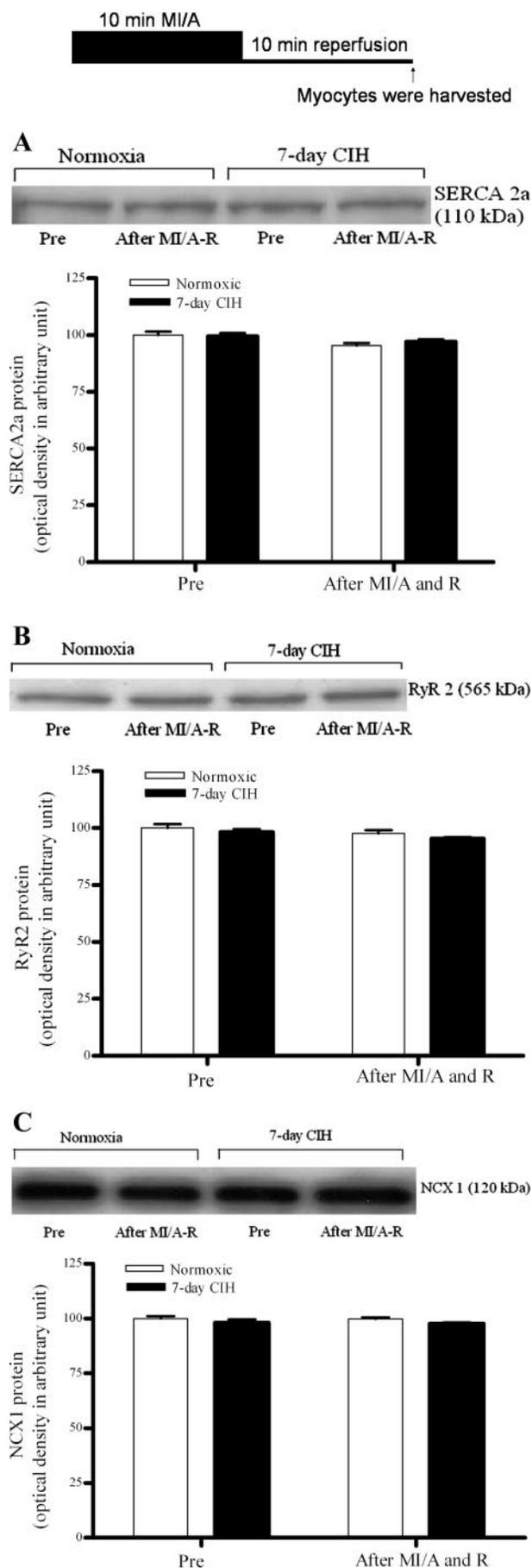


Fig. 5. Effects of CIH on the activities of $^{45}\text{Ca}^{2+}$ flux of SERCA2 (A), ryanodine receptor (RyR) proteins of SR (B), and sarcolemmal Na^+ / Ca^{2+} exchanger (NCX; C) in ventricular myocytes subjected to MI/A and reperfusion. Experimental protocol is the same as in Fig. 6. Values are means \pm SE; $n = 4-6$ rats in each group. * $P < 0.05$ vs. corresponding pretreatment values (Pre). ### $P < 0.001$ and # $P < 0.05$ vs. corresponding normoxic group.

diastolic Ca^{2+} level in the CIH cardiomyocytes during MI/A-R, which is important to the increased myocardial tolerance against I/R injury. In fact, I/R-induced increase in intracellular diastolic Ca^{2+} level indicated by the elevation of resting $[\text{Ca}^{2+}]_i$ level in MI/A-R was significantly less in the CIH groups, supporting an ameliorated Ca^{2+} homeostasis in CIH cardiomyocytes in I/R. Thus, the decrease in amplitudes



of the $E[\text{Ca}^{2+}]_i$ and $C[\text{Ca}^{2+}]_i$ transient of the CIH cardiomyocyte was much less than that of the normoxic control during MI/A-R, meaning an ameliorated maintenance of the cardiac contractility and the Ca^{2+} content of the SR in the CIH cardiomyocyte in I/R. Moreover, the increase in T_{50} values of the $E[\text{Ca}^{2+}]_i$ and $C[\text{Ca}^{2+}]_i$ transient of the CIH cardiomyocyte was much less than that of the normoxic group during MI/A-R, suggesting augmented SR and sarcolemmal function for the ameliorated Ca^{2+} sequestration of the Ca^{2+} content in CIH cardiomyocytes in I/R. Furthermore, the speed of Ca^{2+} release via RyR of SR, indicated by the value of time to peak of $E[\text{Ca}^{2+}]_i$ transients, was significantly faster in the CIH cardiomyocyte in MI/A-R, resulting in augmented Ca^{2+} release from the SR and so better the maintenance of the amplitude of $E[\text{Ca}^{2+}]_i$ transients of CIH cardiomyocytes in I/R. However, the $E[\text{Ca}^{2+}]_i$ is affected by multiple factors for examples the superficial Ca^{2+} level, activities of voltage-gated channels; membrane-bound calmodulin-dependent kinase and Na^+/K^+ pump of sarcolemma. Thus the Ca^{2+} measurement in isolated cells may not solely reflect the activity of Ca^{2+} handling proteins directly measured by the $^{45}\text{Ca}^{2+}$ studies.

Our results showed that ameliorated Ca^{2+} handling in CIH cardiomyocytes in MI/A-R was mainly due to augmented RyR and NCX activities. Thus, the rates of $^{45}\text{Ca}^{2+}$ release via RyR of SR and extrusion by sarcolemmal NCX, instead of uptake by SERCA, were significantly increased in the CIH cardiomyocytes in MI/A-R. The augmented Ca^{2+} extrusion by sarcolemmal NCX could account for the reduction in T_{50} values of the $E[\text{Ca}^{2+}]_i$ and $C[\text{Ca}^{2+}]_i$ transients in I/R. These findings support the hypothesis that changes in Ca^{2+} -handling mechanisms are associated with the CIH-induced cardioprotection. Also results are supportive to the findings reported recently showing that alterations in the activity and expression of SERCA, RyR and NCX are involved in IAH-induced cardioprotection against I/R injury (6). Yet, cellular mechanisms involved could be different between the CIH- and the IAH-induced cardioprotection. Hence, the augmented NCX and RyR activities cannot be attributed to alterations in the amount of protein because the protein expressions of SERCA, RyR, and NCX remained at the same level following MI/A-R in the CIH cardiomyocyte. In addition, the SERCA activity was less affected in the CIH cardiomyocyte. It is plausible that the SERCA activity regulated by phospholamban (PLB) may be different in the CIH-induced cardioprotection. The level of phosphorylation of Ca^{2+} handling proteins in cardiomyocytes varies in pathological conditions and both serine/threonine kinases and phosphatases play important roles in the phosphorylation. So, the regulation of activities of the kinases and phosphatases may be altered differently in the CIH group. Alternatively, the discrepancies could be due to differences in severity of exposure to hypoxia (7 vs. 42 days), specific conditions during preparation of animal models (normobaric vs. hypobaric condition) and also different protocols used in the experimentation of the I/R studies.

Fig. 6. Effects of CIH on the protein expressions of SERCA2 (A), RyR proteins of SR (B), and sarcolemmal NCX (C) in ventricular myocytes subjected to MI/A and reperfusion. Experimental protocol is shown at top. Values are means \pm SE; $n = 4-6$ rats in each group. * $P < 0.05$ vs. corresponding pretreatment values (Pre) and # $P < 0.05$ vs. corresponding normoxic group.

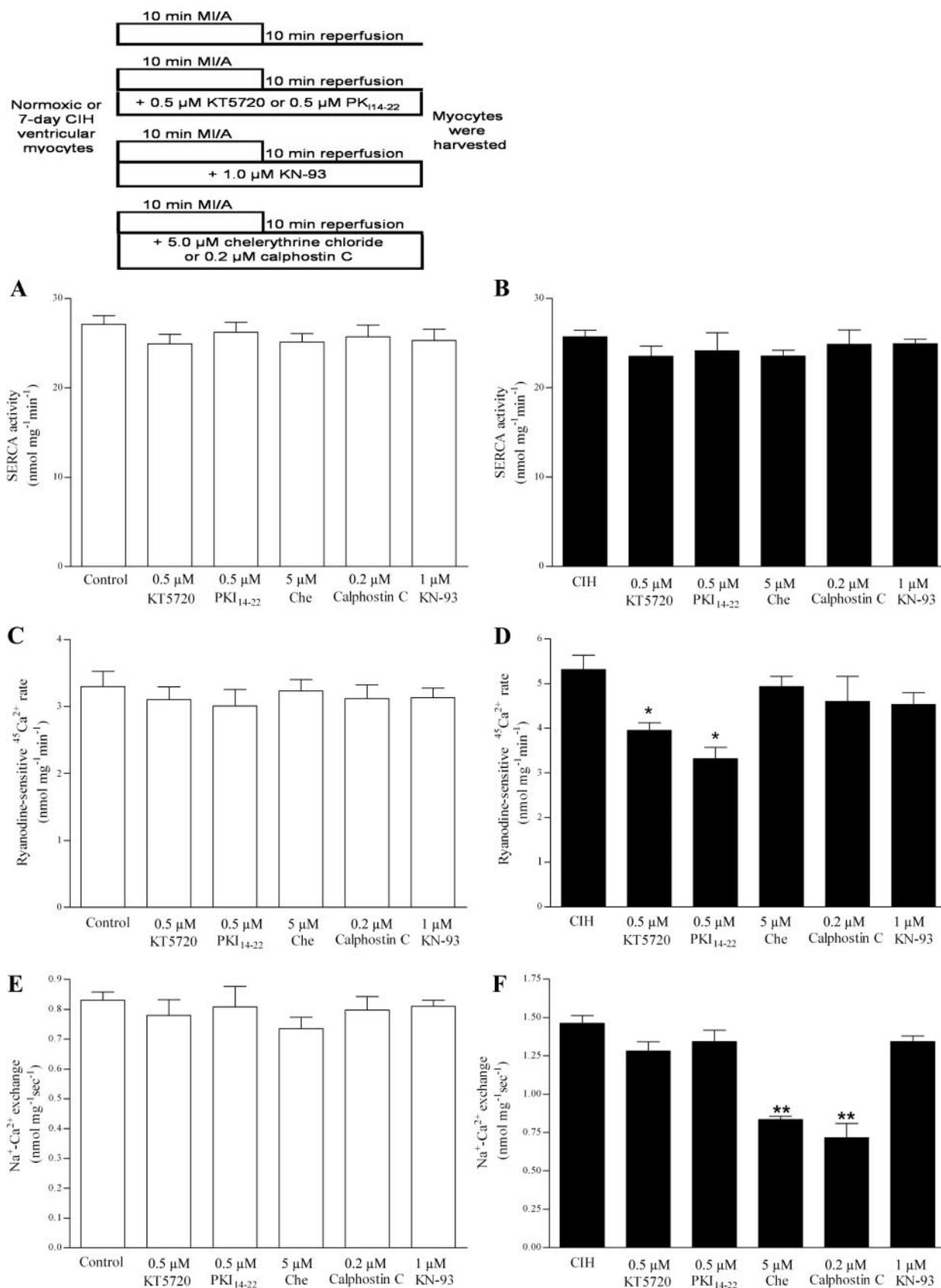


Fig. 7. Effects of CIH on $^{45}\text{Ca}^{2+}$ uptake via SERCA (A and B), release via RyR (C and D) and extrusion by NCX (E and F) upon blockade by corresponding kinase inhibitors of protein kinase A (KT5720 and PKI₁₄₋₂₂), protein kinase C (Che and calphostin C), and Ca^{2+} /calmodulin-dependent protein kinase II (KN-93). Experimental protocol is shown at top. Values are means \pm SE; $n = 5-6$ rats in each group. * $P < 0.05$ vs. corresponding control. ** $P < 0.01$ vs. corresponding control. Che, chelerythrine chloride; PKI₁₄₋₂₂, myristoylated PKA inhibitor amide 14-22, cell permeable.

The fact that the augmented RyR and NCX activity was abolished, respectively, by PKA and PKC inhibitors, suggesting a mechanistic role of PKA and PKC phosphorylation in the CIH-induced cardioprotection. Yet, the SERCA activity in CIH cardiomyocytes was not affected by PKA, PKC, and CaMKII inhibition in MI/A-R. This indicates that additional mechanisms may be involved in the maintenance of the SERCA activity in I/R. In this context, there are many studies showing that decrease in SERCA activity in cardiomyocytes from failing hearts leads to increase in NCX activity to compensate and allow maintenance in normal Ca²⁺ homeostasis (39, 45). It is well known that SERCA is important for the Ca²⁺ sequestration with ATP hydrolysis. It interacts with PLB that reversibly lowers its affinity for Ca²⁺ (41). Study has been shown that acclimatization to IAH activates PKA and CaMKII to phosphorylate PLB and relieve its inhibitory effect on SERCA activity during I/R (47). Thus, it is possible that the regulation and level of phosphorylation of the PLB and Ca²⁺ handling proteins may be altered such that the overall effect on the PLB and/or SERCA is unaffected.

The RyR2 is the most abundant isoform in cardiomyocytes and it is a complex with a large cytoplasmic domain serving as a scaffold for regulatory proteins that modulate the RyR channel activity (24). This domain contains highly conserved leucine/isoleucine zippers to bind the targeting proteins such as PKA and PP1 (35). The RyR2 is tightly bound with four 12.6-kDa binding protein (FKBP12.6) subunits that modulate the channel activity. Each FKBP12.6 contains a phosphorylation site (RyR2-Ser2809) that can be phosphorylated mainly by PKA or CaMKII. When this site is phosphorylated by PKA, the FKBP12.6 is dissociated from RyR2, increasing the open probability and in turn increasing cardiac contractility. Our results clearly showed that PKA inhibition with KT5720 abolished the increased activity of RyR of SR in CIH cardiomyocytes in MI/A-R, suggesting that phosphorylation by PKA is involved in the increase in the RyR activity, contributing to better maintenance of the amplitude of E[Ca²⁺]_i during ischemic insults.

The NCX1 is the main isoform found in cardiomyocytes and it is highly regulated by many cytosolic factors such as cytosolic Na⁺ and Ca²⁺ concentration, MgATP level and intracellular pH (36). It has been shown that phosphorylation of cardiac NCX1 is via PKC-dependent pathway and its fusion protein containing the entire NCX1 cytoplasmic domain (amino acids 240-737) further maps out the phosphorylation sites that can be phosphorylated by PKA, PKC and CaMKII respectively to modulate its activity (15). It has been shown that I/R decreases the phosphorylation of SR and sarcolemmal Ca²⁺ handling proteins to cause abnormalities in cardiac functions (28–30, 43). Our results demonstrated that PKC inhibition with chelerythrine chloride abolished the augmented activity of sarcolemmal NCX in CIH cardiomyocytes in MI/A-R, indicating that phosphorylation by PKC mediates the increase in the NCX activity. This results in the decreased T₅₀ value of the E[Ca²⁺]_i and C[Ca²⁺]_i transients and so fastens the Ca²⁺ extrusion in CIH cardiomyocytes in I/R. The presence of 1 μM KN-93, which can be completely blocked CaMKII activity (17, 20), did not have any effects on SERCA, nor on the activity of RyR of SR and sarcolemmal NCX, suggesting that CaMKII may not play a major role in the CIH-induced cardioprotection. Hence, our results support the hypothesis that phosphorylation

mediated by regulatory kinases of the Ca²⁺ handling proteins plays a mechanistic role of in the augmented Ca²⁺ handling of SR and sarcolemma in the CIH cardiomyocytes.

In conclusion, we have demonstrated that the Ca²⁺ homeostasis was not significantly altered in the heart of rats with 7-day CIH but it confers cardioprotection against I/R injury. We further showed that the cardiac Ca²⁺ homeostasis is ameliorated by augmented activities of RyR of SR and sarcolemmal NCX in I/R. Mechanistically, activation of PKA and PKC plays an important role in the Ca²⁺ handling mechanism involved in the CIH-induced cardioprotection against I/R injury.

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