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N-n-Butyl Haloperidol Iodide Preserves Cardiomyocyte Calcium Homeostasis during Hypoxia/Ischemia

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Key Words

N-n-Butyl haloperidol iodide • Calcium transients • Ventricular myocytes • Hypoxia/ischemia

Abstract

Aims: N-n-Butyl haloperidol iodide (F_2) is a novel compound derived from haloperidol. In our previous work, F_2 was found to be an L-type calcium channel blocker which played a protective role in rat heart ischemic–reperfusion injury in a dose-dependent manner. In the current study, we aimed to investigate the effects and some possible mechanisms of F_2 on calcium transients in hypoxic/ischemic rat cardiac myocytes. Methods and Results: Calcium transients' images of rat cardiac myocytes were recorded during simulated hypoxia, using a confocal calcium imaging system. The amplitude, rising time from 25% to 75% (RT25-75), decay time from 75% to 25% (DT75-25) of calcium transients, and resting $[Ca^{2+}]_i$ were extracted from the images by self-coding programs. In this study, hypoxia produced a substantial increase in diastolic $[Ca^{2+}]_i$ and reduced the amplitude of calcium transients. Both RT25-75 and DT75-25 of Ca^{2+} transients were significantly prolonged. And F_2 could reduce the increase in resting $[Ca^{2+}]_i$ and the prolongation of RT25-75 and DT75-25 of Ca^{2+} transients during hypoxia. F_2 also inhibited the reduction in amplitude of calcium transients which was caused by 30-min hypoxia. The activity of SERCA2a (sarcoplasmic reticulum Ca^{2+}-ATPase, determined by test kits) decreased after 30-min ischemia, and intravenous F_2 in rats could ameliorate the decreased activity of SERCA2a. The inward and outward currents of NCX (recorded by whole-cell patch-clamp analysis) were reduced during 10-min hypoxia, and F_2 further inhibited the outward currents of NCX during 10-min hypoxia. All these data of SERCA2a and NCX might be responsible for the changes in calcium transients during hypoxia. Conclusion: Our data suggest that F_2 reduced changes in calcium transients that caused by hypoxia/ischemia, which was regarded to be a protective role in calcium homeostasis of ventricular myocytes, probably via changing the function of SERCA2a.

Introduction

Cardiac excitation-contraction coupling results from electrical excitation of myocytes to contraction of the heart. Normal calcium signaling is essential in cardiac electrical activity and is the direct activator of myofila-

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ments, which cause contraction. Cardiac myocytes show a transient increase in cytoplasmic $[Ca^{2+}]_i$ during each heartbeat, which is caused by Ca^{2+} influx through voltage-dependent sarcolemmal Ca^{2+} channels during the action potential and ensures Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (SR) through ryanodine receptors (RyRs), the Ca^{2+} release channels. This sudden elevation in $[Ca^{2+}]_i$, which is transient, is required for the development of contractile activity [1]. Myocytes mishandling of Ca^{2+} is a central cause of both contractile dysfunction and arrhythmias in pathophysiological conditions [2]. Previous findings in several species showed that ischemia produced a substantial disorder in diastolic and systolic Ca^{2+}, which was associated with a series of changes in Ca^{2+} transients [3-5].

Haloperidol (Hal) is a typical antipsychotic agent and clinically used for the treatment of psychological disorders such as schizophrenia and mania. Since 1993, our previous research had shown that Hal had effects on vasodilatation and anti-myocardial ischemia. But its side effects on the extrapyramidal system limited large sample observation and further study. So we designed and synthesized a series of quaternary ammonium salt derivatives of Hal. N-n-Butyl haloperidol iodide (F_2) is one of these compounds, it is impossible for F_2 to pass through the blood-brain barrier and the cardiac and vascular effects would hopefully be preserved. F_2 was granted a Chinese national invention patent (No. ZL96119098.1).

In our previous work, we found F_2 to be an L-type calcium channel blocker that inhibited $I_{Ca,L}$ (IC50=0.17μM) in rat ventricular myocytes [6]. F_2 played a protective role in rat heart ischemia–reperfusion injury in a dose-dependent manner, which reduced the release of creatine kinase (CK), creatine kinase isoenzyme MB, lactate dehydrogenase, α-hydroxybutyrate dehydrogenase and glutamic oxalic transaminase, preserved the activity of superoxide dismutase and dose-dependently decreased the malondialdehyde content [6]. F_2 also exerted antiarrhythmic properties in ischemia- and reperfusion-induced arrhythmias [7]. Its cardioprotective mechanisms might be associated with the inhibition of Ca^{2+} overload by blocking the L-type calcium channels of ventricular myocytes [6, 8] and suppressing the expression of early growth response 1 [9, 10]. F_2 also inhibited translocation of PKCα, increased translocation of PKCε, and relieved the CK release and apoptosis in primary cultured cardiomyocytes exposed to H/R [11]. In the current study, we aimed to investigate the effects and some possible mechanisms of F_2 on calcium transients in hypoxic/ischemic rat cardiac myocytes.

Methods and Materials

Myocardial ischemia injury

We used adult male Sprague-Dawley rats (200-250g) that were treated in compliance with The Guide for the Care of Use of Laboratory Animals by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and followed the rules of the National Animal Protection of China. All experimental protocols were approved by the Laboratory Animal Ethics Committee of our institution (No. 2008-045). The study was approved by the Institutional Animal Care and Use Committee of Shantou University Medical College. The model of heart ischemia injury was induced in anesthetized rats as previously described [12]. Rats were randomly assigned to 7 groups (n=8 each) for treatment: sham, the left anterior descending coronary artery was surrounded by a silk thread but not ligated; ischemia, ischemia for 30 min; and intravenous F_2, 0.25, 0.5, 1, 2 and 4 mg/kg before induction of ischemia.

Cardiac myocytes isolation

Single ventricular cardiac myocytes were isolated from the hearts of adult male rats by a standard enzymatic technique [13, 14]. Briefly, after rats were anaesthetized by intraperitoneal injection of trichloroacetaldehyde monohydrate (0.5 g/kg), the heart was removed from the chest, cleaned and flushed with nominally Ca^{2+}-free Tyrode solution consisting of (in mM) 137 NaCl, 5.4 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 10 glucose, and 20 Hepes (pH 7.4) and perfused by use of a Langendorff apparatus. Perfusion began with nominally Ca^{2+}-free Tyrode solution for 5 min at 37°C, and then switched to the enzyme solution with 0.4 mg/mL collagenase (Worthington, Type α) and 0.06 mg/mL protease (Sigma, Type XIV) for 25 min. After perfusion, the heart was minced into small chunks, and single cells were shaken loose from the heart tissue and stored in Hepes-buffered solution containing (in mM): 137 NaCl, 5.4 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 1 $CaCl_2$, 20 glucose and 20 Hepes (pH 7.4, adjusted with NaOH). Cells were used within 8 h after isolation.

Confocal imaging of intracellular Ca^{2+} transients

For confocal Ca^{2+} imaging, freshly isolated ventricular cardiac myocytes were loaded with 5 μM fluo-4 AM (Molecular Probes, USA) for 15 min at room temperature and then washed with a Tyrode solution that contained 2.5 mM Ca^{2+} to remove excess dye. Confocal imaging involved use of an Olympus FluoView FV1000 confocal microscope (Olympus Inc., Japan) equipped with an argon laser (488 nm) and a 40×, 1.3NA objective, at axial and radial resolutions of 1.0 and 0.4 μm, respectively. The experiments were conducted at room temperature. Contractions and Ca^{2+} transients were elicited by field-stimulating myocytes through a pair of platinum electrodes with a 0.5-ms supra-threshold voltage square pulse. Cells were stimulated at 1 Hz to produce steady-state conditions, which was well below the physiological frequency (4-6 Hz). This stimulation was necessary because in most myocytes stimulated at the rate of 4 Hz, the decay of the preceding Ca^{2+} transients was not completed at room temperature. In these myocytes, analyzing the decaying part of Ca^{2+} transients was not precise

enough, which precluded reliable calculation of decay time from 75% to 25% of Ca^{2+} transients (DT75-25) and evaluation of SERCA2a function [15]. The amplitude of electrically induced calcium transients was determined as difference between the resting $[Ca^{2+}]_i$ (F_0) and the peak $[Ca^{2+}]_i$ (F), $\Delta F/F_0 = (F - F_0)/ F_0$.

Model of simulated hypoxia

After 20 min of superfusion with normal Tyrode solution, isolated ventricular cardiac myocytes were superfused in a randomized, blinded fashion to 1 of 4 simulated hypoxia solutions by the addition of stock solution containing 0, 0.1, 1, 10 μM F_2. The 0-μM F_2 stock solution contained only dimethylsulfoxide (DMSO), a solvent of F_2. The simulated hypoxia solution contained (in mM) 123 NaCl, 6 $NaHCO_3$, 0.9 NaH_2PO_4, 8 KCl, 0.5 $MgSO_4$, 2.5 $CaCl_2$, and 20 sodium lactate (pH 6.8; gassed with 90% N_2-10% CO_2) [16, 17]. Superfusion with buffer was controlled by gravity to maintain a flow rate of 6 ml/min.

Ca^{2+} transport by SR Ca^{2+}-ATPase and Na^+/Ca^{2+} exchanger

The elimination of Ca^{2+} from the cytosol resulting in the decaying phase of electrically stimulated Ca^{2+} transients is attributed to the parallel function of SR Ca^{2+}-ATPase (SERCA2a), Na^+/Ca^{2+} exchanger (NCX) and plasmalemmal Ca^{2+}-ATPase (PMCA) working at given rates (V_{SERCA}, V_{NCX}, and V_{PMCA}, respectively). The rate of Ca^{2+} transport by three transporters was estimated from the 50% peak of Ca^{2+} transients' amplitude, decaying time from 75% to 25% (DT75-25). To estimate the rate of Ca^{2+} transport by SERCA2a, the contribution of NCX and PMCA was eliminated. As shown in Fig. 3A, myocytes were incubated with 5μM thapsigargin (TG) blocking SERCA2a uptake of calcium for 6 min after 30-min hypoxia at room temperature [18]. Under these conditions, the rate constant of decline reflects the rate of Ca^{2+} transport by NCX and PMCA ($V_{NCX+PMCA}$). The rate of Ca^{2+} transport by SERCA2a was calculated as $V_{SERCA}=V_{TOTAL} - V_{NCX+PMCA}$.

To estimate the rate of Ca^{2+} transport by NCX, the myocytes were incubated with 5μM TG, 5μM carboxyeosin (CE), a specific PMCA blocker, and 10μM Ru360 to block uptake into mitochondria for 6 min after 30-min hypoxia at room temperature [18]. Therefore, under these conditions, the rate constant of decline reflects the rate of Ca^{2+} transport by NCX (VNCX).

Isolation of SR and measurement of SERCA2a activity

We cut the infracted myocardium from myocardium of apex heart which color changed to dark from the surrounding tissue. SR vesicles were obtained as described previously [19, 20] with some modification. Briefly, freshly isolated rat left ventricles were washed in ice-cold 0.9% NaCl and homogenized in an extraction medium containing (in mM) 15 Tris HCl, 10 $NaHCO_3$, 5 NaN_3, 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 8 ml of a mixture of 0.6 mM 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 and a Teflon pestle and stored at 70°C. All solutions contained 3 protease inhibitors: aprotinin (48μg/ml), pepstatinA (5μM) PMSF (1mM), and leupeptin (48 μg/ml). The activities of SERCA2a were determined by use of test kits (Jiancheng Bioengineering Inst., Nanjing, China) as described [21]. One unit of SERCA2a activity is defined as the quantity of ATP enzyme degrading 1 μmol phosphorusprothour; the data are expressed as SERCA2a activity per gram of tissue.

Western blot analysis

Total protein extracts were prepared from myocardial tissue cells with use of cell lysis buffer containing a protease inhibitor cocktail (aprotinin, leupeptin, pepstatin A, and PMSF). Equal amounts of total protein (60 μg for tissue) underwent 10% SDS-PAGE, and then were electrophoretically transferred to nitrocellulose membranes. Nonspecific binding was blocked by incubation of membranes with skim milk overnight at 4°C. The blot was incubated with a rabbit anti-rat RyR, SERCA2a or NCX antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were then washed with TBS-T and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000) at room temperature for 1 h. Detection of immunoreactive bands involved chemiluminescence luminol reagents (Pierce Biotechnology, USA). Blots were then reprobed with actin (1:2000, Sigma-Aldrich, USA) to confirm equal loading of samples.

Whole-cell patch-clamp and bi-directional NCX current recording

Whole-cell patch clamp recording of the single ventricular myocytes was as described [7]. The patch-clamp amplifier was Axopatch 200B (Axon Instruments, Foster City, CA, USA). For patch clamp recordings, the extracellular solution contained (in mM) NaCl 140, $CaCl_2$ 2, $MgCl_2$ 1, and HEPES 10 (pH was adjusted to 7.4). To block Na^+/K^+ pump currents and currents flowing through K^+ or Ca^{2+} channels, ouabain (0.02mM), $CsCl_2$ (2 mM), $BaCl_2$ (2 mM) and nifedipine (0.01mM) were added to the solution. The pipette solution contained (in mM) CsOH 120, aspartic acid 50, NaCl 20, $CaCl_2$ 10 (free Ca^{2+} concentration 226nM), BAPTA 20, $MgCl_2$ 3, MgATP 5, and HEPES 10.

Ramp pulses were given every 10 s from the holding potential of -60 mV initially depolarizing to 30 mV and then hyperpolarizing to -150 mV and depolarizing back to -60 mV with a constant speed of 0.72 Vs^{-1}. The descending limb of the pulse was used to obtain the data [7, 22]. First the descending limbs (from +30 to -150mV) which plotted as the I-V relationship without capacitance compensation were obtained before hypoxia; Hypoxia condition was made by perfusing the cell with 10-min hypoxia solution and the current-voltage (I-V) recorded. Then perfusing the cell with hypoxia+1μM F_2 solution and the current-voltage (I-V) recorded; finally a high concentration of 5mM Ni^{2+} was applied to completely block I_{NCX} and the current-voltage (I-V) recorded. This method is convenient, fast and severe enough to produce a hypoxia single cell model [23]. The tem-

perature of all perfusing solutions was maintained at room temperature.

Statistical analysis

Results are expressed as mean plus or minus the standard error of the mean (SEM). Statistical analysis was performed using the Student t-test for unpaired samples; a value of $P < 0.05$ was considered significant.

Results

The effect of F_2 on calcium transients during hypoxia

We first assessed the effect of F_2 during acute hypoxia on the basic characteristics of calcium transients as measured in our experimental system. The resting F_0 represented the diastolic cytosolic Ca^{2+} content during the calcium transients. The overall resting F_0 in the line-scan image slowly increased during hypoxia as compared with the control. The resting F_0 increased from 100% to 145.4±4.08% (P<0.05) after 30-min perfusion of hypoxia solution, which showed a significant increase in diastolic $[Ca^{2+}]_i$ in hypoxia. The resting F_0 increased from 100% to 122.01±0.84%, 119.14±0.65% and 118.71±3.12% after perfusion with hypoxia solutions containing 0.1, 1 and 10 μM F_2 respectively (Fig. 1, Fig. 2A). These data showed that F_2 could reduce the increase in resting $[Ca^{2+}]_i$ which was caused by 30-min hypoxia.

Time to peak calcium transients represents the rate of Ca^{2+} release from the SR, mainly via RyRs. We used the rising time from 25% to 75% (RT25-75) during the time to peak of calcium transients to estimate the rate of Ca^{2+} release from the SR. After 30-min hypoxia, the RT25-75 significantly prolonged from 100% of control to 247.70±6.21% (P<0.05). The increase in RT25-75 suggests that hypoxia could reduce RyR activity. In cells treated with 0.1, 1 or 10 μM F_2during hypoxia, RT25-75 was 193.24±3.27%, 178.82±2.45% or 176.55±1.52%, respectively (Fig. 1, Fig. 2B). These data showed that F_2 could reduce the prolongation of RT25-75 of Ca^{2+} transients during hypoxia.

After the excitation–contraction coupling, Ca^{2+} must be removed from the cytosol to lower $[Ca^{2+}]_i$ and allows relaxation. The decay of calcium transients represented the course of Ca^{2+} transport out of the cytosol by 4 pathways involving SERCA2a, sarcolemmal NCX, sarcolemmal Ca^{2+}-ATPase and mitochondrial Ca^{2+} uniporter. In rat ventricular myocytes, the SERCA2a pump removes 92% of the activator Ca^{2+}, and NCX removes 7%, leaving only about 1% each to be removed by the sarcolemmal Ca^{2+}-ATPase(PMCA) and mitochondrial Ca^{2+} uniporter [24]. SERCA2a is the main factor contributing to the removal of Ca^{2+} from the cytosol during calcium transients in rat cardiac myocytes. Therefore, we measured the 50% decay time from 75% to 25% (DT75-25) of calcium transients. In cells after 30-min hypoxia, the DT75-25 increased to 137.06±1.32% as compared with the control. In cells treated with 0.1, 1 or 10 μM F_2 during 30-min hypoxia, the DT75-25 was 125.70±0.98%, 118.89±0.82% or 114.88±0.64%, respectively (Fig. 1, Fig. 2C). These data showed that F_2 could reduce the prolon-

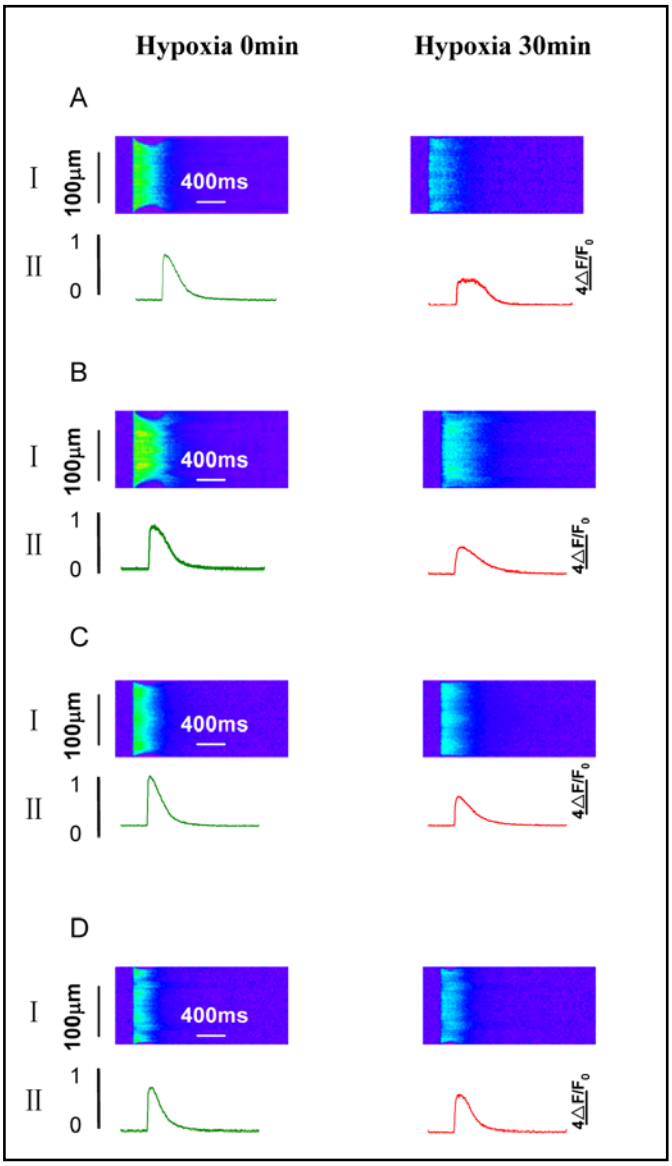

Fig. 1. I. Representative line-scan images of cardiac calcium transients from one single cell perfused with hypoxia solutions. II. Representative time course of cardiac calcium transients, derived from line scan images in I. Left bar in II: normalized amplitude scale. A, B, C, D represent hypoxia, hypoxia +0.1 μM F_2, hypoxia +1 μM F_2 and hypoxia +10 μM F_2.

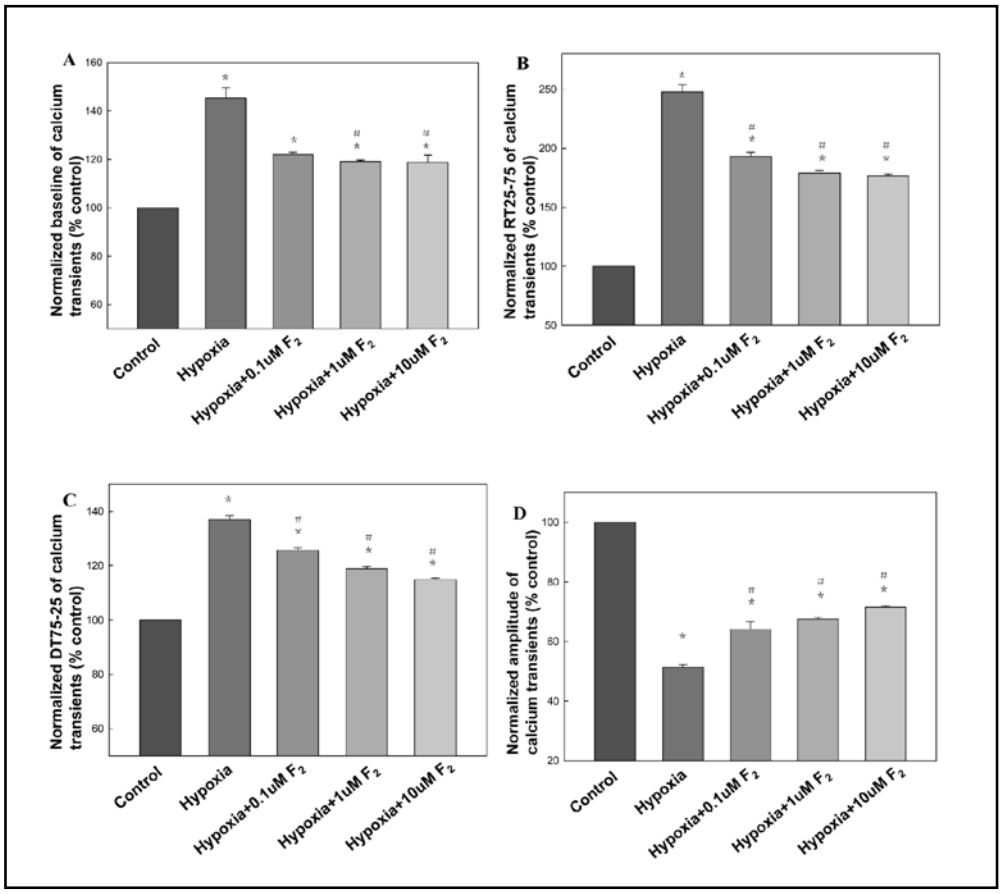

Fig. 2. Change in (A) baseline, (B) rising time from 25% to 75% (RT25-75), (C) decay time from 75% to 25% (DT75-25) and (D) peak amplitude of calcium transients perfused with hypoxia solutions or hypoxia+F_2 solutions. Data are means ±SE (n=18 cells per group). *P<0.05, compared with control. #P<0.05 compared with hypoxia 30 min.

gation of DT75-25 of Ca^{2+} transients during hypoxia.

To estimate the rate of Ca^{2+} transported by SERCA2a, the contribution of NCX and PMCA was eliminated. As shown in Fig. 3A, myocytes were incubated with 5μM thapsigargin (TG), blocking SERCA2a uptake of calcium, for 6 min after 30-min hypoxia at room temperature. Under these conditions, the rate constant of decline reflects the rate of Ca^{2+} transport by NCX and PMCA ($V_{NCX+PMCA}$). In cells after 30-min hypoxia, the V_{SERCA} decreased to 25.97±1.82% as compared with normal V_{TOTAL}. In cells treated with 0.1, 1 or 10 μM F_2 during 30-min hypoxia, the decreasing V_{SERCA} was dose-dependently reduced to 35.52±2.84%, 38.93±3.69% or 43.89±2.38%, respectively (Fig. 3).

To estimate the rate of Ca^{2+} transport by NCX, myocytes were incubated with 5μM TG and 5μM carboxyeosin (CE), a specific PMCA blocker, for 6 min after 30-min hypoxia at room temperature. However, V_{NCX} did not differ with hypoxia, hypoxia+0.1 μM F_2, hypoxia+1 μM F_2 or hypoxia+10 μM F_2 (Fig. 4).

The amplitude of calcium transients is related to level of resting $[Ca^{2+}]_i$, calcium influx through the L-type calcium channels and the calcium content of SR, and also connected to the activity of RyR and SERCA2a. So any change in these factors could affect the amplitude of calcium transients. The amplitude of calcium transients was reduced to 51.36±3.05% during 30-min hypoxia as compared with the control (P<0.05). In cells treated with 0.1, 1 or 10 μM F_2 during 30-min hypoxia, the amplitude of calcium transients was reduced to 64.13±2.29%, 67.49±3.10% or 71.50±2.16%, respectively (Fig. 1, Fig. 2D).

Effect of F_2 on the activity of SERCA2a during ischemia

To better understand how F_2 affects the decay time of calcium transients during hypoxia, we measured the activity of SERCA2a after 30-min ischemia. As compared with sham treatment, with 30-min ischemia, SERCA2a was reduced to 44.29±7.42% (P<0.05). Intravenous F_2 at 0.25, 0.5, 1, 2 and 4 mg/kg before ischemia reduced SERCA2a to 58.41±5.78%, 66.49±6.10%, 69.49±5.91%, 72.04±5.69% and 83.24±4.77%, respectively (Fig. 6).

Effect of F_2 on NCX inward and outward currents during hypoxia

According to previous study [25], although the total contributions of sarcolemmal NCX, sarcolemmal Ca^{2+}-ATPase, and mitochondrial Ca^{2+} uniporter during the de-

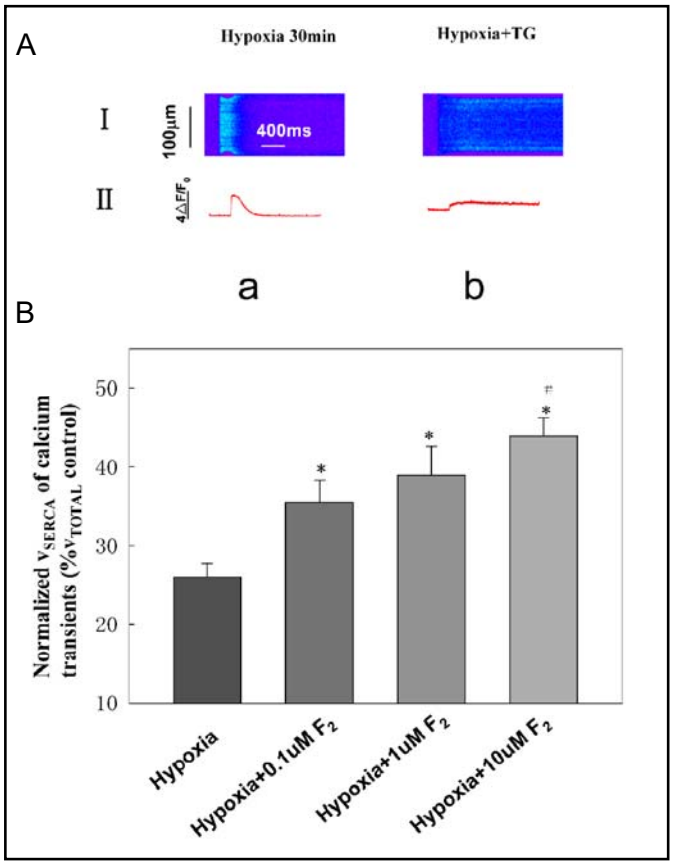

Fig. 3. A I. Representative line scan images of cardiac calcium transients from one single cell perfused with hypoxia solutions. II. Representative time course of cardiac calcium transients, derived from line scan images in I: representative calcium transients after 30-min hypoxia b: representative calcium transients after incubation with 5 μM thapsigargin (TG) to block sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERC2A) uptake, 6 min. B Normalized rate of Ca^{2+} transport by SERCA2a (V_{SERCA}) in calcium transients perfused with hypoxia solutions (%V_{TOTAL} control). Data are means ±SE (n=8 cells per group). *P<0.05, compared with hypoxia. #P<0.05 compared with hypoxia+0.1 μM F_2.

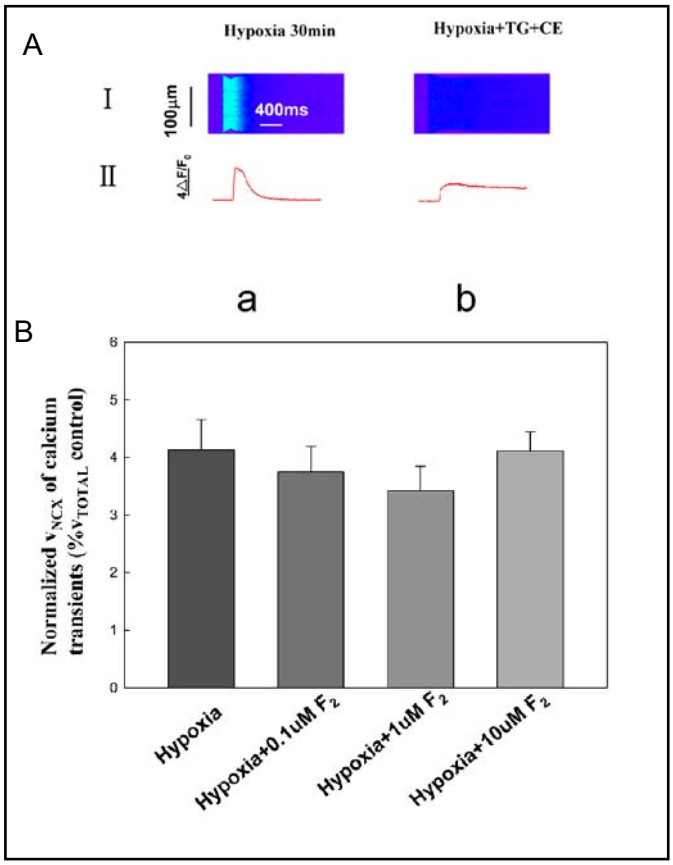

Fig. 4. A I. Representative line scan images of cardiac calcium transients from one single cell perfused with hypoxia solutions. II. Representative time course of cardiac calcium transients, derived from line scan images in I. representative calcium transients after 30-min hypoxia b: representative calcium transients after incubation with 5 μM TG and 5 μM carboxyeosin (CE), a specific PMCA blocker for 6 min after 30-min hypoxia at room temperature. B. Normalized V_{NCX} in calcium transients perfused with hypoxia solutions (%V_{TOTAL} normal). Data are means ±SE (n=8 cells per group).

cay time of calcium transients is little, NCX removes 7% Ca^{2+} during the Ca^{2+} removal from the cytosol to lower $[Ca^{2+}]_i$ and allows relaxation in rat ventricular myocytes. Whether F_2 has direct effects on NCX during the Ca^{2+} removal from the cytosol to lower $[Ca^{2+}]_i$ and allows relaxation in rat ventricular myocytes during hypoxia is unclear. Therefore, we sought to determine the effect of F_2 on NCX inward and outward currents during hypoxia. Compared with control currents, the inward and outward currents of NCX were reduced to 41.28% and 47.22%, respectively after 10-min hypoxia. With the hypoxia solution containing 1 μM F_2, the inward and outward currents of NCX were reduced to 38.15% and 15.45%, respectively (Fig. 5).

Effect of F_2 on the protein levels of RyR, SERCA2a and NCX during ischemia

To better understand how F_2 affects the intracellular Ca^{2+} and Ca^{2+} dynamics during hypoxia, we measured the protein levels of RyR, SERCA2a and NCX after 30-min ischemia. As shown in Fig. 7, the protein level of SERCA2a and NCX did not change after 30-min ischemia as compared with the control. In addition, with intravenous F_2 at 0.25, 0.5, 1, 2 and 4 mg/kg before the induction of ischemia, the protein level of RyR did not change after 30-min ischemia.

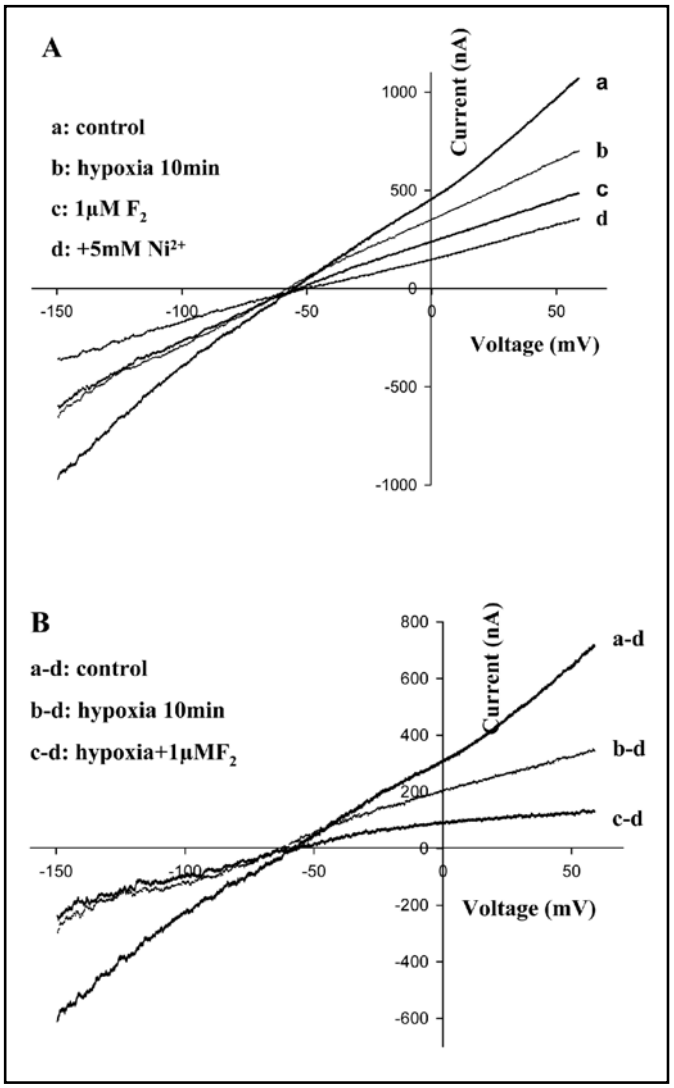

Fig. 5. Effect of F_2 on Na^+/Ca^{2+} currents after 10-min hypoxia. A: +60 to -150 mV ramp impulse induced Na^+/Ca^{2+} exchanger (NCX) currents perfused with hypoxia for 10 min; B: a-d: Ni^{2+}-sensitive NCX currents at control; b-d: Ni^{2+}-sensitive NCX currents after 10-min hypoxia; c-d: Ni^{2+}-sensitive NCX currents at hypoxia+1 μM F_2.

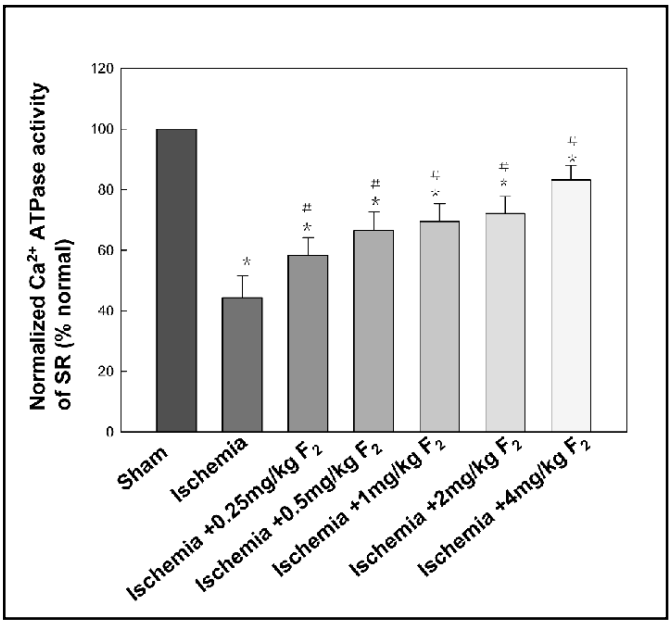

Fig. 6. F_2 preserved the reduction in normalized activity of SERCA2a with ischemia for 30 min. Data are means ±SE (n=8 rats per groups). *p<0.05, compared with sham group. #p<0.05 compared with 30-min ischemia group.

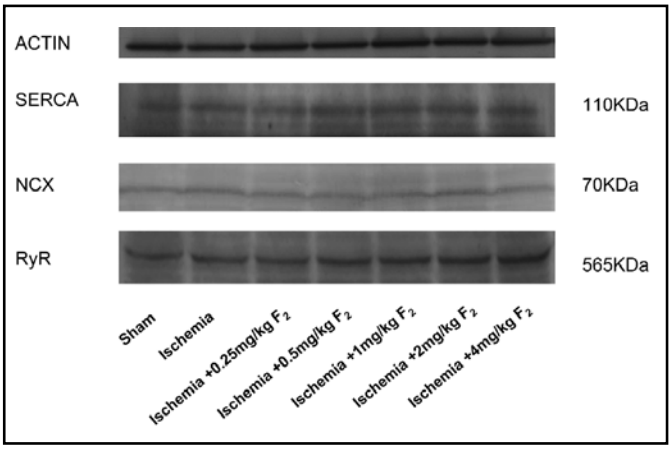

Fig. 7. Effect of ischemic insult on the protein levels of SERCA2a, NCX and ryanodine receptor (RyR).

Discussion

We aimed to study the effects of F_2 on calcium transients and related factors in hypoxic/ischemic rat cardiac myocytes and explore the protective mechanisms and targets of F_2 on ischemia–reperfusion injury in rats. First, we observed the changes in calcium transients in ventricular myocytes during hypoxia and then observed simultaneously the changes in activity of the proteins SERCA2a and NCX, which are related to calcium transients. Consistent with previous findings[17, 26], we confirmed the series of changes occurring in calcium transients during hypoxia/ischemia, which include an increase in RT25-75 and DT75-25 of Ca^{2+} transients, accompanied by the amplitude reducing and increasing of basal Ca^{2+} level of calcium transients during 30-min hypoxia.

Previous studies showed that F_2 is an L-type calcium channel blocker that inhibited $I_{Ca,L}$ in rat ventricular myocytes[7, 8]. In this study on the effects of F_2 on calcium transients and related factors during hypoxia/ischemia, we found the amplitude of calcium transients reduced during 30-min hypoxia, which could be partly inhibited by F_2. The amplitude of calcium transients is mainly related to factors that include calcium influx through

L-type calcium channel (LTCC), the resting $[Ca^{2+}]_i$, the Ca^{2+} load in SR (associated with the activity of SERCA2a), and the activity of RyR and SERCA2a. In this study we found although the expression of SERCA2a did not change significantly, the activity of SERCA2a was reduced, which could decrease the calcium load of the SR during hypoxia/ischemia. The increase in resting $[Ca^{2+}]_i$ could be augmented by hypoxia/ischemia-induced depolarization of the membrane potential, which allows for opening the LTCC and further entry of Ca^{2+} into the cell [27]. All of these factors were associated with reduced calcium transients amplitude during 30-min hypoxia. F_2 played a protective role on the activity of SERCA2a during 30-min hypoxia/ischemia. However, why F_2 could inhibit the reduction of calcium transient amplitude during 30-min hypoxia, when F_2 could block the LTCC on the cardiac ventricular and seemed to have no direct effect on RyR. F_2 possibly protects the activity of SERCA2a during 30-min hypoxia (Fig. 8). Previous studies showed an increase in amplitude of calcium transients during acidosis [28]. The reason for this discrepancy may be connected with the difference in the observation time, the degree of acidosis and hypoxia models. For example, during acidosis, glucose is not replaced by sodium lactate [28].

The decay of calcium transients represents the course of Ca^{2+} transported out of the cytosol by 3 pathways, including SERCA2a, sarcolemmal NCX, and sarcolemmal Ca^{2+}-ATPase [24, 25]. We found the DT75-25 of calcium transients prolonged after 30-min hypoxia, whereas treatment with 0.1, 1 or 10 μM F_2 during 30-min hypoxia dose-dependently reduced the prolongation of DT75-25. In rat ventricular myocytes, the SR Ca^{2+}-ATPase pump removes 92% of the activator Ca^{2+}, and NCX removes 7%, leaving only about 1% each to be removed by the sarcolemmal Ca^{2+}-ATPase and mitochondrial Ca^{2+} uniporter [24]. Because of the minimal contribution of PMCA on calcium transients, and the pre-test showed no change in PMCA during hypoxia, we did not consider the effect of PMCA on calcium transients. SERCA2a is primarily responsible for SR Ca^{2+} uptake and replenishing the SR Ca^{2+} load during the contraction–relaxation cycle of the heart. The SR shows substantial damage with impaired Ca^{2+} uptake during ischemia–reperfusion [27]. This finding agrees with our results of the V_{SERCA} of calcium transients reduced and the activity of SERCA2a decreased during hypoxia/ischemia, but the protein level of SERCA2a did not change. Treatment with 0.1, 1 or 10 μM of F_2 during 30-min hypoxia dose-dependently inhibited the reduction of V_{SERCA}, which agrees with results of

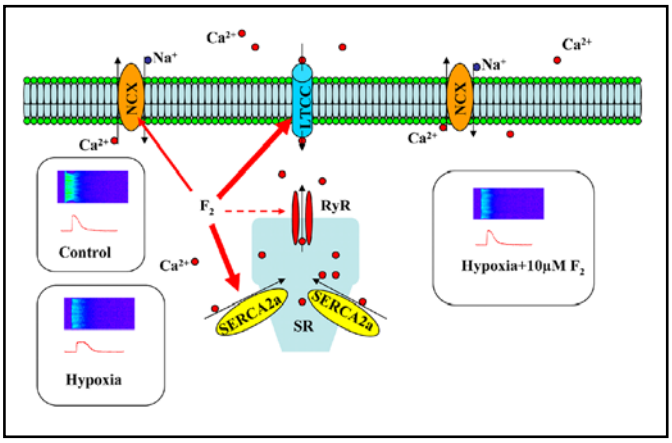

Fig. 8. Possible mechanism of F_2 during hypoxia/ischemia. Membrane depolarization opening the L-type calcium channel leads to calcium overload during hypoxia. The activity of NCX, SERCA2a is decreased during hypoxia/ischemia. F_2 blocks the L-type calcium channel and plays a protective role in the activity of SERCA2a. Whether F_2 directly affects the activity of RyR is unclear.

intravenous treatment with F_2 at 0.25, 0.5, 1, 2 and 4 mg/kg before induction of ischemia, and the damage of SERCA2a was reduced. All of these findings suggest that the activity of SERCA2a reduction during 30-min hypoxia and protective role of F_2 are the major components for the changes (Fig. 8).

The activities of NCX decreased during hypoxia. The inward and outward currents of NCX decreased during 10-min hypoxia. With the hypoxia solution containing 1 μM F_2, the inward and outward currents of NCX also decreased during 10-min hypoxia and the outward currents of NCX decreased more than did the inward currents during 10-min hypoxia. The inhibition of F_2 on the outward currents of NCX may prevent the calcium overload caused by reversal of NCX during hypoxia/ischemia. However, we observed no change in NCX on the decay time of calcium transients and the effects of F_2 on the NCX during 30-min hypoxia. This finding may be connected with the little effect of F_2 on the inward currents of NCX and contribution of NCX on the decay time of calcium transients or with the time of hypoxia.

Calcium was necessary for excitation-contraction coupling but also acted as a second message in ventricular myocytes and connected with PKC. PKCα could upregulate PP-1, render phospholamban hypophosphorylated, and promote stronger inhibition of SERCA2a [29]. Our previous study showed that F_2 inhibiting translocation of PKCα may be connected with the prevention of calcium overload by inhibiting $I_{Ca,L}$ after hypoxia-reoxygenation [11]. Therefore, we deduced that

protection of SERCA2a activity by F_2 after 30-min hypoxia might, in part, be a result of the reduction of Ca^{2+} overload-mediated translocation of PKCα. Cytosolic Ca^{2+} concentration was regulated precisely by SERCA2a, NCX, and the slow systems. The increasing $[Ca^{2+}]_i$ would activate the SERCA2a uptaking more Ca^{2+} into the SR by Ca^{2+}/calmodulin-dependent protein kinase α during hypoxia/ischemia [30], which leads to more energy metabolism and inhibits the SERCA2a activity in feedback, thus aggravating intracellular calcium overload. Therefore, protection of SERCA2a activity by F_2 after 30-min hypoxia may be connected with reducing energy metabolism. Nevertheless, whether a Ca^{2+}/PKCα/SERCA2a/Ca^{2+} positive-feedback loop exists during hypoxia-reoxygenation needs further exploration.

Study showed that the reduction in amplitude of calcium transients and the inhibition of RyR activity and Ca^{2+}-ATPase of SR during hypoxia increasing the propensity of calcium transients alteration [31]. Calcium overload and calcium transient alterations during ischemia play causal roles in the genesis of arrhythmias [31, 32]. Our results indicated that F_2 could reduce the change in calcium transients caused by hypoxia, which may be related to antiarrhythmic properties in ischemia-and reperfusion-induced arrhythmias we found previously [7].

Ca^{2+} transients is considered the summation of many microscopic Ca^{2+} release events triggered by one or more single Ca^{2+} currents [33]. Abnormalities in intracellular calcium handing are widely recognized as a common hallmark of heart disease in animal models and humans [34, 35]. Study on the trigger mechanisms and characteristics of calcium transients help us understand the pathophysiology mechanisms of heart diseases. So F_2 could prevent calcium overload by blocking the LTCC in cardiac ventricular myocytes during hypoxia/ischemia. As well, the effect of F_2 on calcium transients (including the amplitude and the DT75-25) was mainly targeted to the activity of SERCA2a during hypoxia/ischemia. Our study on the effect of a calcium channel blocker about calcium transients and related factors during hypoxia will help us explore new mechanisms of calcium channel blockers and provide a new theoretical basis for the development of new drugs.

The intracellular ions changes are complex and correlated during hypoxia/ischemia. A lot of studies have shown Ca^{2+}, Na^+ and the generation of H^+ increased during hypoxia. The intracellular ions maintain a dynamic state balance. For example, the generation of protons are extruded from ventricular myocytes via Na^+-H^+ exchange (NHE), Ca^{2+} are removed to extracellular via Na^+-Ca^{2+} exchanger (NCX) and Ca^{2+} also can enter the ventricular myocytes via NCX reverse mode. [36, 37]. F_2 could reverse calcium overload during hypoxia by inhibiting L-type calcium currents, so F_2 may also influence pH and $[Na^+]_i$ during hypoxia directly or indirectly. But the effect of F_2 on pH and Na^+ during hypoxia still need further study.

Our study contains some limitations. Our findings apply to field-stimulated rat ventricular myocytes paced at 1 Hz and investigated at room temperature. Whether similar observations would be obtained in cells stimulated at physiological frequency (e.g., 4 Hz) and temperatures is unknown. According to other lab previous study [17, 38], we found that the change of calcium transient is more sensitive during hypoxia than reperfusion compared with control, so we observed the effects of F_2 on calcium transients during 30-min hypoxia. As well, we observed the changes in inward and outward currents for NCX only after 10-min hypoxia, not 30-min hypoxia to ensure the survival of ventricular myocytes.

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