Introduction

The myocardium consists of various cell types, including cardiomyocytes, smooth muscle cells, endothelial cells, and fibroblasts. Among them, about 30% of the cells are composed of myocytes and the rest of the cells are non-myocytes (1). Fibroblasts are major non-muscle cells in the heart, which reside in the extracellular matrix (ECM), and they synthesize and secrete fibrillar collagen type I and III (2). The ECM forms a three-dimensional network that surrounds bundles of myocytes and contributes to transmission of mechanical force generated by contraction of cardiac myocytes. Cardiac fibroblasts are important not only in the healthy state, but also under pathological conditions, such as hypertension, myocardial infarction, and heart failure, which induce cardiac remodeling (3, 4).

In myocardial infarction, cardiac tissue is exposed to ischemia by coronary artery stenosis or embolism. If the area at risk remains hypoxic, the myocardium loses its contractile function, becomes necrotic, and fibrotic changes are initiated via the wound-healing process (4, 5). Cardiac fibroblasts play an important role in this process. For example, hypoxia triggers the differentiation of fibroblasts to myofibroblasts (6), leading to their matrix metalloprotease (MMP)-dependent invasion (7) with collagen synthesis (8) and resulting cardiac remodeling. Thus, hypoxia is a pathophysiological stimulus for cardiac fibroblasts. Although cardiac fibroblasts are electrically “non-excitable” cells, various membrane currents were found in rat (9 – 13) and human cardiac fibroblasts (14), including an inward rectifier K+ current, a delayed rectifier K+ current, and a non-selective cation current. However, the roles of those currents are not fully understood in the function of cardiac fibroblasts.

Hypoxia is a major pathological stress for the cells in general, but the effect of hypoxia on ion channel expression is unknown in cardiac fibroblasts. In this study, we investigated the effect of hypoxia on ion channels in cardiac fibroblasts using various methods including the whole cell patch-clamp technique, pharmacological inhibition, RNA interference, and quantitative RT-PCR.

Hypoxic Stress Induces Transient Receptor Potential Melastatin 2 (TRPM2) Channel Expression in Adult Rat Cardiac Fibroblasts

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Abstract. When cardiac tissue is exposed to hypoxia, myocytes are damaged, while fibroblasts are activated. However, it is unknown what changes are induced by hypoxia in cardiac fibroblasts. In this study, using the whole cell patch-clamp technique, we investigated the effect of hypoxia on membrane currents in fibroblasts primarily cultured from adult rat hearts. Cardiac fibroblasts were incubated for 24 h under normoxic or hypoxic conditions using Anaeropack. Hypoxia increased a current which reversed at around −20 mV in the cardiac fibroblasts. This current was inhibited by clotrimazole, which is an inhibitor of transient receptor potential melastatin 2 (TRPM2) channel and intermediate-conductance Ca2+-activated K+ channel (KCa3.1). ADP ribose in the pipette solution enhanced this current. Quantitative RT-PCR revealed that mRNA of TRPM2, but not that of KCa3.1, was increased by hypoxia. RNA interference of TRPM2 prevented the development of the hypoxia-induced current. H2O2, an activator of TRPM2 channel, induced a higher [Ca2+]i elevation in hypoxia-exposed cardiac fibroblasts than that in normoxia-exposed cells. We conclude that hypoxia induces TRPM2 channel expression in adult rat cardiac fibroblasts.

Keywords: hypoxia, cardiac fibroblast, rat heart, clotrimazole, transient receptor potential melastatin 2 (TRPM2) channel
Materials and Methods

Ethical approval

All experiments were performed with the approval of the Animal Research Committee of Fukushima Medical University.

Isolation and culture of rat cardiac fibroblasts

Adult rat cardiac fibroblasts were isolated and cultured as described previously (13, 15, 16) with modification. In brief, male Wistar rats (8 – 14-week-old) were anesthetized with ether. The heart was excised and the atria and valves were removed. The ventricles were minced, and incubated in Ringer solution containing 0.3% collagenase (Wako, Tokyo) for 45 min at 37°C. The Ringer solution contained 137 mM NaCl, 8.1 mM NaHCO3, 2.7 mM KCl, 1.2 mM KH2PO4, 1 mM CaCl2, 0.5 mM MgCl2, and 10 mM glucose (pH 7.4). After centrifugation, the supernatant was extracted and suspended in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS), 0.001% penicillin-G (Banyu Pharmaceutical, Tokyo), and streptomycin (Meiji Seika, Tokyo). Cardiac fibroblasts were separated from myocytes by selective cellular attachment. The cell suspension incubated in an uncoated plastic culture dish (100 mm) for 60 min at 37°C to allow for the preferential attachment of fibroblasts, after which unattached cells were rinsed off. Cardiac fibroblasts were maintained in a humidified atmosphere of 5% CO2 at 37°C and used at passage 1 – 3. Within these passages, no difference was recognized in morphology and responses of the cells to hypoxia. It was reported with this method that the purity of cultures at these passages was > 95% fibroblasts (15).

Preparation of hypoxic conditions

After a day of seeding on coverslips, cardiac fibroblasts were divided into two groups: one group was incubated under normoxic and another group, under hypoxic conditions for 24 h before electrophysiological analysis. Hypoxic conditions were created by using an Anaeropack (Mitsubishi Gas Chemicals, Tokyo), which contained sodium ascorbate as the principal ingredient, to absorb oxygen and generate carbon dioxide by oxidative degradation (17, 18). The culture dishes containing fibroblasts were placed in an airtight jar with an Anaeropack and incubated at 37°C for 24 h. In the jar, the oxygen concentration was expected to decrease to a level less than 1% within 1 h and carbon dioxide concentration was maintained at about 5% (18). After 24 h, hypoxia was terminated by opening the Anaeropack jar, and the fibroblasts in the culture dishes were taken out and incubated in a CO2 incubator at 37°C for 30 min. The experiments were initiated within 0.5 – 2 h after stopping the hypoxic incubation.

Patch-clamp experiment

Membrane currents were recorded from the cardiac fibroblasts by the whole-cell patch clamp method (19). The recording chamber was attached to the stage of an inverted microscope (Model 80121; Nikon, Tokyo). Cells were superfused with Tyrode solution at a rate of 1 ml/min. The temperature of the bath solution was maintained at 36°C ± 0.5°C with a water jacket. Patch pipettes were forged from glass capillaries with 1.5-mm internal diameter and 2.1-mm external diameter (Nihon Rikagaku Kikai, Tokyo) using a microelectrode puller (pp-83; Narishige, Tokyo). Pipette resistance was 2 – 4 MΩ when filled with the pipette solution. The pipette solution consisted of 100 mM K-glucuronate, 20 mM KCl, 16 mM KOH, 1 mM MgCl2, 1 mM CaCl2, 11 mM EGTA, 5 mM ATP-2Na, 16 mM KOH, and 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] (pH 7.2 with KOH). ADP ribose was added to the pipette solution to a final concentration of 0.3 mM. Tyrode solution contained 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 0.33 mM NaH2PO4, 5 mM HEPES, and 5.5 mM glucose (pH 7.4 with 2.4 mM NaOH). The electrode was connected to a patch-clamp amplifier (CEZ-2400; Nihon Kohden, Tokyo). Recording signals were filtered at 2.5-kHz bandwidth, and the series resistance was compensated. Current signals were stored online and analyzed using pClamp Version 9 (Axon Instruments, Union City, CA, USA). Square voltage pulses of 500-ms duration were given with a 10-mV step increment every 10 s from the holding potential (HP) of −40 mV over the voltage range between −70 and 40 mV. The current magnitude was measured at the end of the 500-ms pulse for current-voltage relation analysis.

Total RNA isolation and quantitative RT-PCR

Total RNA was extracted from the cells using the QIAzol™ Lysis Reagent (Qiagen, Tokyo), Qiagen RNeasy™ Mini Kit™ (Qiagen) with the QIAcube™ (Qiagen) following the manufacturer’s protocol. Reverse transcription of mRNA into cDNA was carried out using the iScript™ cDNA synthesis kit (Bio-Rad, Tokyo). Complementary DNA preparation was then stored at −20°C until further use. Messenger RNA levels were quantified using a quantitative reverse transcriptase PCR (qRT-PCR) system (iCycler iQ, Bio-Rad). PCR reactions were performed with the iQ™ SYBR® Green Supermix (Bio-Rad), using 2.5 μL of cDNA as a template in each 25 μL reaction mixture. The PCR protocol was as follows: an initial 10 s denaturation at 95°C followed by 40 cycles of 5 s of denaturation at 96°C, 10 s of annealing at 60°C, and 30 s of extension at 72°C. Then the PCR
samples were cooled to 55°C, 10 s. After that, the samples were heated to 95°C (0.05°C/s) for the analysis of dissociation curves in the iCycler. Then the PCR samples were cooled to 40°C. All qRT-PCR reactions were run in triplicate. The sequence of the primers and predicted lengths of the PCR products are summarized in Table 1. The primers for transient receptor potential melastatin 2 (TRPM2) were designed as described previously (20). Other sets of primer pairs were available commercially (Takara Bio, Ohtsu). As for endogenous controls, we employed ribosome 18S (r18S). The PCR products were checked by the Bioanalyzer (Agilent Technologies, Tokyo), and the specificities of the primers were confirmed by the dissociation curves and their first derivatives, each of which giving only one peak.

For quantification of cDNA, we used iCycler software version 3.0A (Bio-Rad). To generate a standard curve, the cDNA template that was mixed with all samples in each experiment was diluted four times. We adopted a six-point standard curve in each of the amplification experiments for normalization, ranging from 1/64 to 1/2 of the cDNA template. Dilutions were done with deionized water.

RNA interference

Double-stranded 25-bp small interfering (si) RNAs to the selected region of TRPM2 mRNA (Stealth Select RNAi) were purchased from Invitrogen (Tokyo). The siRNA sequences targeting rat TRPM2 are summarized in Table 2. Negative control siRNA (Medium GC Duplex, Invitrogen) was used as a control. Cardiac fibroblasts were transfected with siRNA (30 nM) using PrimaPort transfection reagent (Credia-Japan, Kyoto) according to the manufacturer’s instructions. We harvested cells at 48 – 96 h after transfection.

Ca²⁺ imaging

To evaluate the Ca²⁺ response, cardiac fibroblasts were plated on glass-bottomed dishes (Matsunami, Tokyo) in DMEM with 10% FBS. After 24 h under the hypoxic condition, cells were loaded with 3 μM Fura-2AM (Dojindo, Kumamoto) in Tyrode solution for 25 min at 37°C. Measurement of Fura-2 fluorescence was performed with the Aquacosmos-Ratio imaging system (Hamamatsu Photonics, Hamamatsu). Image acquisition was maintained at a frequency of one image every 5 s and stored in a computer with Aquacosmos imaging software (Hamamatsu Photonics). Excitation wave lengths employed were 340 and 380 nm, and the emis-

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### Table 1. Oligonucleotide primers used for PCR amplification of cDNAs

<table>
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<tr>
<th>Protein</th>
<th>Gene</th>
<th>Accession No.</th>
<th>Primer sequences</th>
<th>Length (bp)</th>
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<td>Trpm2</td>
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<tr>
<td>KCa3.1</td>
<td>Kcnm4</td>
<td>NM_023021</td>
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<tr>
<td>TGF-β1</td>
<td>Tgfb1</td>
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<td>82</td>
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<tr>
<td>p21</td>
<td>Cdkn1a</td>
<td>NM_080782</td>
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<tr>
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<tr>
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<td>140</td>
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### Table 2. Sequences of siRNA oligonucleotide used

<table>
<thead>
<tr>
<th>Name</th>
<th>Target gene (accession No.)</th>
<th>Primers (5′-3′)</th>
<th>GC contents (%)</th>
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</thead>
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<td>Trpm2-RSS309198</td>
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<tr>
<td>Trpm2-RSS309199</td>
<td>TRPM2 (NM_001011559)</td>
<td>AUCUCAGCCCGCAUAUGAGGUCCG&lt;br&gt;CGACCUCAUCUUGACGGCGGUAU</td>
<td>52</td>
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<td>Trpm2-RSS309200</td>
<td>TRPM2 (NM_001011559)</td>
<td>UAGCCGUGUAGCUUCUCUCUCGCGAC&lt;br&gt;GCGUGCAGAAGCGAAGGAACAUA</td>
<td>52</td>
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sion wavelength was 510 nm. The fluorescence ratio (F340/F380) within an image area of an individual cell was calculated. Extracellular solutions were heated so that the temperature in a recording chamber was maintained at 37°C.

Drugs
Clotrimazole (CLT, Wako), diisothiocyanatostilbene disulfonic acid (DIDS; Sigma, Tokyo), and 4-aminopyridine (4-AP, Sigma) were first dissolved in DMSO and added to the bath solution. The final concentration of DMSO was 0.1% or less. For control experiments, 0.1% DMSO alone was applied. Tetraethylammonium (TEA, Wako) was dissolved in water. H2O2 (Wako) was diluted in Tyrode solution.

Analyses
All values are presented as mean ± S.E.M. The percent inhibition of the current magnitude was measured at 40 mV for various CLT concentrations. The relationship between the concentration of CLT and the rate of inhibition (I) was fitted by the following equation:

\[
I = \left[ \frac{(I_{\text{max}} - I_{\text{min}})}{(1 + (IC_{50} / [CLT]))} \right] + I_{\text{min}},
\]

where \(I_{\text{max}}\) is the maximum value (which equals control conditions), \(I_{\text{min}}\) is the minimum value (which was calculated by curve fitting software: Origin version 6.1; OriginLab Corp., Northampton, MA, USA), IC_{50} is the median value of the concentration–inhibition curve, and \(n\) is the Hill coefficient. [CLT] indicates the CLT concentration. Statistical significance between the two groups or among multiple groups was evaluated using Student’s \(t\)-test, Tukey’s test, or Dunnett’s test after the F-test or one-way ANOVA.

Results

Effects of hypoxia on whole-cell current in rat cardiac fibroblasts
We first examined whether hypoxic stress affected membrane currents in cardiac fibroblasts. To do so, we recorded the whole-cell currents from cultured cardiac fibroblasts exposed to normoxia or hypoxia for 24 h with voltage pulses between −70 and 40 mV from the holding potential of −40 mV. It has been reported that cardiac fibroblasts express multiple ion channels (14). In the present study, several types of currents were observed in different fibroblasts. Figure 1, A and B, illustrates a typical set of currents from normoxia and hypoxia-exposed cells. The current which reversed at around −20 mV was significantly increased in both the outward and inward direction in hypoxia-exposed cells compared to those of normoxia-exposed ones. This type of current was observed with similar frequency in both groups: 8 out of 14 normoxia-exposed cells (57.1%) and 13 out of 23 hypoxia-exposed ones (56.5%). Other types of currents include the one that reversed at more negative potentials, possibly a K+ current, and another that showed a marked time-dependent decay similar to a transient outward K+ current. The magnitude of these types of currents was not significantly different between normoxia and hypoxia. Thus, we selected currents that reversed at around −20 mV without time-dependent decay. The current amplitude was measured at the end of 500-ms square pulses at each potential and the average current density was plotted as current–voltage (I-V) relationships (Fig. 1C). The mean current density from hypoxia-exposed cells was significantly decreased at −70 mV and increased in the range of 10–40 mV (\(P < 0.05\)) (Fig. 1C). In hypoxia-exposed cells (\(n = 13\)), inward current at −70 mV and outward current at 40 mV were increased to 297% and 182%, respectively, of those in normoxia-exposed control cells (\(n = 8\), Fig. 1D).

The average resting potential was −23.0 ± 8.1 mV (\(n = 8\)) in the normoxic cells, and it was −20.4 ± 2.8 mV (\(n = 13\)) in hypoxia-exposed cells. Therefore, hypoxia did not change the resting potential of the fibroblasts (\(P > 0.05\)). The membrane capacitance was also not altered by hypoxia (data not shown). Thus, we concluded that hypoxia increased the membrane current density in rat cardiac fibroblasts.

Effect of CLT on hypoxia-induced current in rat cardiac fibroblasts
To identify the current enhanced by hypoxia, we tested several pharmacological inhibitors of Cl− channels or nonselective cation channels because the hypoxia-induced current reversed at around −20 mV. A Cl− channel inhibitor, DIDS (100 μM), did not inhibit the current (data not shown), indicating that the current is not a Cl− current.

CLT is an inhibitor of a non-selective cation current through the TRPM2 channel (21, 22). When we tested 10 μM CLT, the current in normoxia-exposed fibroblasts were only slightly inhibited (Fig. 2: A, B, and C). However, as shown in Fig. 2D–F, CLT significantly decreased both the inward current at −70 mV and the outward current at 40 mV in hypoxia-exposed cells (\(n = 3–5, P < 0.05\)). The inhibition of CLT was irreversible. Figure 2G illustrates the averaged I-V curves of the net CLT-sensitive current density calculated from Fig. 2, C and F. The average voltage where the CLT-sensitive current reversed was at −19.4 ± 5.0 mV (\(n = 5\)). This value coincided with the resting potential of the hypoxia-induced cells (Fig. 1). The concentration–response curve was obtained by applying various concentrations of CLT in the range from 10 nM to 10 μM (Fig. 2H). The percent inhibition was calculated at 40 mV for each CLT concen-
A sigmoid curve fitting yielded a median inhibitory concentration (IC$_{50}$) of 0.7 μM (Fig. 2H).

We performed an additional experiment using ADP ribose and could successfully record an augmentation of the current by ADP ribose. As shown in Fig. 2I, in hypoxia-exposed cells dialyzed with the pipette solution containing ADP ribose (0.3 mM), an inward current gradually increased and reached a peak effect by 5 min. Similar results were observed in three other hypoxia-exposed cells. The average maximum increase of the inward current was 1.93-fold of the initial current of the measurement (n = 4). The increase was significant compared to those of other groups (P < 0.05 with Tukey’s test). In normoxia-exposed control cells without ADP ribose, the maximum current became only 1.07-fold after 5 min (n = 4). ADP ribose increased the current significantly only in hypoxia-exposed cells. Thus, we confirmed that the hypoxia-induced current is mediated via TRPM2 channels.

The K$^+$-channel inhibitors, including 4-AP (1 mM) and TEA (5 mM) for voltage-dependent K$^+$ channels and glibenclamide (10 μM) for ATP dependent K$^+$ channels, failed to inhibit the hypoxia-induced current (data not shown). These results indicated that the hypoxia-induced current is mediated via TRPM2 channels.

**Effect of hypoxia on whole-cell current in rat cardiac fibroblasts**

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**Effect of hypoxia on TRPM2 mRNA expression in rat cardiac fibroblast**

Besides TRPM2 channels, CLT is also known to inhibit the intermediate conductance Ca$^{2+}$-activated K$^+$
Hypoxia Increases TRPM2 in Fibroblasts

Therefore, we next examined the mRNA expression levels of TRPM2 channels and KCa3.1 in cardiac fibroblasts exposed to hypoxia and normoxia. In addition, we also examined the expression levels of marker mRNAs for myofibroblasts, for example, transforming growth factor (TGF)-β1 (24), cyclin-dependent kinase inhibitor 1 (p21), and α-smooth muscle actin (α-SMA) (25), because hypoxia is known to induce differentiation from fibroblasts to myofibroblasts (6). As illustrated in Fig. 3, hypoxia significantly increased mRNA levels of TRPM2 (191.8% of the control), while TGF-β1 and p21 mRNAs were decreased to 57.4% and 60.3% of the control, respectively (n = 5 – 6). KCa3.1 and α-SMA mRNA levels were not significantly different between normoxia- and hypoxia-exposed cells (n = 5 – 6). These suggested that the hypoxia-induced current was

Fig. 2. Effect of clotrimazole (CLT) on the current in rat cardiac fibroblasts. A and B: Representative set of currents from a normoxia-exposed cell (A) and those in the presence of 10 μM CLT (B). The voltage steps are the same in Fig. 1A. C: Averaged isochronal I-V curves plotted from normoxia-exposed cells in the absence (open diamonds, n = 5) and presence of 10 μM CLT (closed diamonds, n = 3). The current magnitude was measured at the end of 500-ms pulses. Data are indicated as the mean ± S.E.M. D and E: Representative currents recorded from fibroblasts exposed to hypoxia in the absence (D) or presence (E) of 10 μM CLT. F: Averaged isochronal I-V curves plotted from hypoxia-exposed cells in the absence (open squares, n = 3 – 5) or presence of 10 μM CLT (closed squares, n = 3 – 5). The current magnitude was measured at the end of 500-ms pulses. Data are indicated as the mean ± S.E.M. G: CLT-sensitive I-V curves obtained by the difference between a pair of I-V curves in C (normoxia; open diamonds, n = 3) and between those in F (hypoxia; closed diamonds, n = 3 – 5). Data are indicated as the mean ± S.E.M. H: Concentration-inhibition relationships between CLT and currents at 40 mV and different concentrations of CLT in hypoxia-exposed cells (closed squares). At 10 μM CLT, inhibition was compared with that in normoxia (open square) (**P < 0.01, Student’s t-test). The IC50 value of CLT was 0.7 μM. I: Effect of ADP ribose (0.3 mM) in the pipette solution on the current recorded at −70 mV from fibroblasts. A voltage step pulse of 500-ms duration from a holding potential of −40 mV was applied every 10 s. Representative current densities during 5 min are plotted. Normoxia control without ADP ribose (open triangles), normoxia with ADP ribose (open squares), hypoxia control without ADP ribose (closed triangles), and hypoxia with ADP ribose (closed squares).
Hypoxia induced an increase of TGF-β1 mRNA, which was most effective (Fig. 4: A and B), and tested whether they inhibit TRPM2 mRNA expression in cardiac fibroblasts. We designed three siRNAs for TRPM2, and used siRNA to deplete the TRPM2 expression levels of TRPM2 mRNA at 96 h after transfection of siRNA (n = 3 – 4, Fig. 4A). Since DIDS (100 μM), a Cl− channel inhibitor, did not inhibit the hypoxia-induced current, the possibility of Cl− channels was negated.

Effect of siRNA for TRPM2 on hypoxia-induced current

To verify that the hypoxia-induced current was TRPM2, we used siRNA to deplete the TRPM2 expression in cardiac fibroblasts. We designed three siRNAs and tested whether they inhibit TRPM2 mRNA expression in hypoxia-exposed cells. In our hands, two siRNAs (RSS-309199 and -309200) significantly decreased the expression levels of TRPM2 mRNA at 96 h after transfection of siRNA (n = 3 – 4, Fig. 4A). Since RSS-309200 was most effective (Fig. 4: A and B), we used this TRPM2 siRNA for our further study. TRPM2 siRNA induced an increase of TGF-β1 mRNA, but KCa3.1 and p21 were unaffected (Fig. 4B). When the whole-cell current was measured from the fibroblasts transfected with RSS-309200, a marked reduction in the current was seen even after 24-h hypoxia (Fig. 4: C and D). The average I-V relationships were obtained from fibroblasts with control siRNA (n = 3) or TRPM2 siRNA (n = 4 – 5, Fig. 4E). Both inward current at −70 mV and outward current at 40 mV were significantly smaller in TRPM2 siRNA-transfected cells (n = 4 – 5, Fig. 4F). The average resting potentials were not significantly different between the control and TRPM2 siRNA–infected cells (P > 0.05). These results further support our conclusion that hypoxia induced TRPM2 channel expression in cardiac fibroblasts.

Discussion

Effect of H2O2 on [Ca2+]i in hypoxia-exposed rat cardiac fibroblasts

The TRPM2 channel is a Ca2+-permeable nonselective cation channel. It has been reported that TRPM2-channel activity is augmented by H2O2 (26 – 28). If the hypoxia-induced current is TRPM2, H2O2 should elevate [Ca2+]i, more strongly in hypoxia-exposed cells than normoxia-exposed ones. We tested it with the Fura-2 fluorometric technique. The basal [Ca2+]i in hypoxia-exposed cells tended to be higher than that in normoxia-exposed ones, although no statistical significance was obtained. H2O2 at 300 μM increased [Ca2+]i, in both normoxia- and hypoxia-exposed cells. The increase in the Fura-2 ratio was higher in hypoxia-exposed fibroblasts (n = 23) than in normoxia-exposed ones (n = 20, Fig. 5A). Figure 5B summarizes the area under the curve (AUC) of Fura-2 ratio in the presence of H2O2, which was significantly greater (P < 0.01) in hypoxia-exposed cells (Fig. 5B). These results further supported our conclusion that hypoxia-induced TRPM2 channel expression in cardiac fibroblasts.

We could not obtain calibration of the fluorescence signals in terms of Ca2+ concentrations because many cells were detached from the bottom of the culture dish when we added 1 mM EGTA (without Ca2+) or 1.8 mM Ca2+ after permeabilization of cells with 10 μM iomycin at the end of the experiment.
known to inhibit KCa3.1 (23), the free Ca$^{2+}$ concentration in our pipette solution was calculated to be about 13 nM, which was too low to activate KCa3.1 that requires free Ca$^{2+}$ concentration higher than 200 nM (31). Hill et al. reported in HEK293 cells expressing TRPM2 that CLT at 3 – 30 μM completely and irreversibly inhibited the TRPM2 current, which was activated by ADP-ribose in the pipette solution (21). In the present study, we found that 10 μM CLT completely and irreversibly inhibited the hypoxia-induced current. By testing CLT for 5 min at each concentration over the range 0.01 – 10 μM, we obtained the IC$_{50}$ value of 0.7 μM CLT. Hill et al. could not obtain the IC$_{50}$ value of CLT because of the slow rate of block and fragility of the cells (21). They reported that the time to half block at 1 μM CLT was 310 ± 58 s, but was 54 ± 13 s at 5 μM CLT, and that it was impossible to

**Fig. 4.** Effect of siRNA for TRPM2 on hypoxia-induced current in rat cardiac fibroblasts. A: TRPM2 mRNA levels in cardiac fibroblasts at 96 h after transfection with three TRPM2 siRNAs (RSS-309198, -309199, and -309200). Data are normalized with 18S rRNA (r18S) and indicated as the mean ± S.E.M. (n = 3 – 4) (*P < 0.05, **P < 0.01: compared to the control, Dunnett’s test). B: Effects of siRNA (RSS-309200) on TRPM2 mRNA in hypoxia-exposed cells. Cells were transfected with RSS-309200 at 24-h before hypoxia and harvested after 24-h hypoxia. Data are normalized to r18S and indicated as the mean ± S.E.M. (n = 4). (**P < 0.01: compared to control, Student’s t-test). C and D: Representative set of currents from a hypoxia-exposed cell transfected with control siRNA (C) and RSS-309200 (D). The voltage steps are the same as in Fig. 1. E: Averaged isochronal I-V curves plotted from hypoxia-exposed cells transfected with control siRNA (open squares, n = 3) and TRPM2 siRNA (RSS-309200) (closed squares, n = 4 – 5). The current magnitude was measured at the end of 500-ms voltage pulses. Data are indicated as the mean ± S.E.M. F: Current densities at −70 and 40 mV. Data are indicated as the mean ± S.E.M. (*P < 0.05, **P < 0.01: compared to the control, Student’s t-test).
determine the level of equilibrium block at a lower concentration because prolonged activation of TRPM2 channels routinely compromises cell integrity, presumably through a combination of intracellular Ca\textsuperscript{2+} and Na\textsuperscript{+} overload (21). In our experiment without ADP-ribose in the pipette solution, the cells survived CLT application for 3 – 5 min and the IC\textsubscript{50} value of 0.7 \(\mu\)M CLT we obtained did not contradict the result of Hill et al. (21).

ADP-ribose is a well-known activator of TRPM2 channels (27). We added ADP ribose in the pipette solution and confirmed that the current gradually and significantly increased only in hypoxia-exposed cells, but not in normoxia-exposed cells (Fig. 2I). Therefore, we hypothesized that non-selective cation current through TRPM2 channels was increased by hypoxia in rat cardiac fibroblasts.

Supporting our hypothesis, we found that hypoxia significantly increased the mRNA levels of TRPM2 but not KCa3.1 in cardiac fibroblasts (Fig. 3). Furthermore, siRNA of TRPM2 decreased both mRNA expression levels of TRPM2 and the membrane current induced by hypoxia (Fig. 4). It has been reported that hypoxia up-regulated various TRP channel expressions, such as TRPC1, TRPC4, and TRPC6, at both the mRNA and protein levels (32, 33). Therefore, TRPM channel expressions may also be regulated by hypoxia in a similar manner to TRPC (TRP canonical) channels.

Hypoxia is known to induce differentiation from fibroblasts to myofibroblasts (6). However, mRNA levels of myofibroblast markers, such as TGF-\(\beta\)1, p21, and \(\alpha\)-SMA, were not increased in our hypoxia-exposed cells. This implies that within the time course of our protocol, the fibroblasts were not yet differentiated to myofibroblasts. Perhaps we harvested the cells too early for the differentiation to initiate. Thus, morphological changes of cardiac fibroblasts by hypoxia may require longer time of reoxygenation and an increase in \([\text{Ca}^{2+}]\). We examined whether the knockdown of TRPM2 induced changes in TGF-\(\beta\)1 and p21 mRNA levels because increases in TGF-\(\beta\)1 and p21 mRNA levels were reported to be signs of myofibroblast formation from fibroblasts. In the present experiment using TRPM2 siRNA, a small but significant increase of TGF-\(\beta\)1 mRNA (1.25-fold of control siRNA) was observed, but p21 mRNA was unaltered (Fig. 4B). We thought, however, that this change in TGF-\(\beta\)1 mRNA must not be due to myofibroblast formation because the change was so small.

In human cardiac fibroblasts, hypoxia decreased basal levels of protein and DNA synthesis in general, but it enhanced growth factor-induced DNA synthesis and basal level of collagen type I (8). Similar to collagen type I in human fibroblasts, TRPM2 mRNA was increased by hypoxia in rat cardiac fibroblasts. This mechanism of increase may be explained by the action of transcriptional factors that respond to hypoxia. In fact, it has been reported that hypoxia inducible factor 1 (HIF-1), a low level under normoxia, is elevated rapidly during hypoxia and increases the expression of TRPC1 and TRPC6 in rat pulmonary arterial smooth muscle cells (33). TRPM2 channels might also be regulated by HIF-1 in cardiac fibroblasts.

The TRPM2 channel is a non-selective cation channel with little preference among various cations. Ca\textsuperscript{2+} permeability ratio against Na\textsuperscript{+} \((P_{\text{Ca}^{2+}}/P_{\text{Na}^{+}})\) is about 0.71 in TRPM2 channels (34). Hill et al. found that replacement of external Ca\textsuperscript{2+} to Ba\textsuperscript{2+} eliminated the TRPM2 current (21). The TRPM2 channel is activated by H\textsubscript{2}O\textsubscript{2}, and TRPM2-mediated Ca\textsuperscript{2+} influx is augmented by H\textsubscript{2}O\textsubscript{2} (26 – 28).

Indeed in our experiment, the increase in \([\text{Ca}^{2+}]\) induced...
by H$_2$O$_2$ in the hypoxia-exposed cells was larger than that in the normoxia-exposed ones. In our study, [Ca$^{2+}$], rise induced by H$_2$O$_2$ started immediately and it took 2 – 3 min to reach a maximal level. This time course is similar to that previously reported in human TRPM2 expressed in HEK cells under similar experimental conditions (28). Unexpectedly, however, CLT did not inhibit H$_2$O$_2$-induced [Ca$^{2+}$] increase, but rather increased it (data not shown). It was reported that CLT inhibited Ca$^{2+}$-ATPase (SERCA) 1 and SERCA2 (35, 36). This may explain why CLT did not inhibit H$_2$O$_2$-induced [Ca$^{2+}$]; increase in our hypoxia-exposed fibroblasts. We suggest that hypoxia-induced current is via TRPM2 nonselective cation channels in cardiac fibroblasts.

TRPM2 is a member of the melastatin-family of transient receptor potential channels found ubiquitously [e.g., central nervous system (26, 37), ventricular myocytes (38), endothelial cells (39), pancreatic β-cells (26), monocytes (40), and immune cells (41, 42)]. However, fibroblasts expressing TRPM2 channels have been so far reported only in the symptomatic dental pulp (43). Thus, our study may be the first report of TRPM2 in rat cardiac fibroblasts.

Because the TRPM2 channel is enhanced by oxidative stress, its function is thought to be a sensor for oxidative stress. Hypoxia induced Ca$^{2+}$ overload and death in rat neonatal cardiomyocytes (44), pancreatic β-cells (45), rat brain striatal cells (46, 47), monocytes (40), and endothelial cells (39). However, in fibroblasts, oxidative stress seems to cause an opposite response. Reactive oxygen species (ROS) activates cardiac fibroblasts to stimulate proliferation (48), collagen synthesis (8), and progression of remodeling in the heart. Thus, hypoxia-induced TRPM2 expression and Ca$^{2+}$ influx may contribute to these processes in cardiac fibroblasts. However, the mechanism of fibroblast activation by increased Ca$^{2+}$ influx is unclear. In fibroblasts from mouse skin, intracellular Ca$^{2+}$ signaling may contribute to cell proliferation and collagen production (49). In human atrial fibroblasts, TRPM7-shRNA reduced Ca$^{2+}$ influx and inhibited proliferation and differentiation induced by TGF-β1 (50). Thus, non-selective TRP cation channels may play a key role in Ca$^{2+}$ influx, which induces activation of fibroblasts and consequently fibrosis.

We conclude that hypoxia induces TRPM2 channel expression in adult rat cardiac fibroblasts. ROS enhances TRPM2 channel activation and thus increases Ca$^{2+}$ entry via TRPM2 channels in cardiac fibroblasts. This may be important in the activation of fibroblasts under cardiac pathological conditions (3, 4). Since TRPM2 channels may make a new therapeutic target for cardiac remodeling, further investigation is necessary for the mechanism of TRPM2 expression under hypoxia in cardiac fibroblasts.

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