Pathophysiological Remodeling of Mouse Cardiac Myocytes Expressing Dominant Negative Mutant of Neuron Restrictive Silencing Factor

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Background: It has been previously reported that the transgenic mouse expressing the dominant negative mutant of the neuron restrictive silencing factor (dnNRSF) in the heart died from lethal arrhythmia, so the present study aimed to clarify the electrophysiological alteration of the ventricular myocyte isolated from the dnNRSF mouse.

Methods and Results: The action potential (AP) and membrane currents were recorded using the whole-cell patch-clamp method. Intracellular Ca^{2+} was measured with Indo-1AM. The AP of dnNRSF myocytes exhibited reduction of resting membrane potential, prolongation of AP duration, and frequent early afterdepolarization (EAD). The EAD was completely inhibited by SEA0400, a specific blocker of the Na^{+} – Ca^{2+} exchanger (NCX). The most notable alteration of membrane current was a reduction in the inward rectifier K^{+} current (I_{K1}) density. In addition to re-expression of fetal type cardiac ion channels, a Na^{+} -permeable, late inward current was observed in a small population of dnNRSF myocytes. The diastolic intracellular Ca^{2+} concentration was also raised in dnNRSF myocytes, and spontaneous Ca^{2+} oscillation was induced by β -adrenergic stimulation.

Conclusions: In dnNRSF myocytes, the “repolarization reserve” of the AP was significantly reduced by specific alterations in membrane currents. Under these conditions, the amplitude of EAD generated by the inward NCX current might be enlarged, thereby increasing the cells’ vulnerability to ventricular arrhythmia. (*Circ J* 2010; **74**: 2712–2719)

Key Words: Arrhythmia; Cardiac myocytes; Hypertrophy; REST/NRSF

Ventricular arrhythmias are a major cause of sudden death in patients with heart failure or cardiac hypertrophy. A characteristic genetic alteration in heart failure appears to be reactivation of fetal cardiac genes, such as atrial natriuretic peptide (ANP), α -skeletal actin, β -myosin heavy chain, and ion channels, including the hyperpolarization-activated, cyclic nucleotide-gated cation channels (I_{h} ; Hcn2; Hcn4) and T-type Ca^{2+} channels ($I_{\text{Ca-T}}$; *Cacna1h*). However, the mechanisms of lethal arrhythmias still remain unclear.^{1–4} We have previously reported that a transcriptional silencer, REST/NRSF (RE-1 silencing transcription factor/neuron restrictive silencing factor) played an important role in transcriptional regulation of ANP and Hcn4 in vitro.^{5,6} In accordance with this, the expressions of ANP, Hcn2, Hcn4, *Cacna1h* and transient receptor potential channel (*Trpc1*) were significantly increased in transgenic mice carrying the dominant negative mutant of REST/NRSF in their heart

(dnNRSF).^{7–9} The dnNRSF mice were vulnerable to ventricular arrhythmia, and started to die suddenly at approximately 8 weeks after birth without showing symptoms of pumping failure, and blockade of $I_{\text{Ca-T}}$ by efonidipine rescued them from sudden death.^{7,10} In a previous study, we reported that the action potential (AP) waveform was significantly different between dnNRSF mice and their wild-type (WT) littermates.¹⁰ Because the ionic mechanisms underlying such alteration of the AP are not clear at present, in the present study we investigated the electrophysiological properties of single ventricular myocytes isolated from dnNRSF mice, aiming to get an insight of the cellular mechanism of arrhythmias.

Methods

All experiments were approved in advance by the Animal Ethics Committee of Jichi Medical University. The investi-

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Figure 1. Action potential (AP) wave forms of ventricular myocytes isolated from wild-type (WT) and dnNRSF mice. **(A)** AP recorded using the perforated patch method. Black line, AP of WT; red line, AP of dnNRSF; blue line, APs recorded in the presence of 10 μ M SEA0400. The dashed line indicates 0 mV. For comparison, the resting membrane potential (RMP) of WT myocyte is shown by the dotted line. **(B)** AP recorded with the ruptured patch method. The pipette solution contained 5 mmol/L EGTA. The AP wave forms were almost superimposable in the presence (blue lines) and absence (WT, black line; dnNRSF, red line) of SEA0400. **(C-a)** Summary of RMP measured with the perforated patch method. RMP of WT was -82.7 ± 3.1 mV (n=15); RMP of dnNRSF was significantly depolarized (-77.8 ± 3.0 mV, n=15) ($P < 0.05$). **(C-b)** RMP measured with the ruptured patch method. RMP of WT, -81.7 ± 1.10 mV (n=15); RMP of dnNRSF, 77.1 ± 0.8 mV (n=15) ($P < 0.05$). **(D-a)** Duration of 90% AP (APD₉₀) measured with the perforated patch method. APD₉₀ of dnNRSF was significantly longer (148.5 ± 14.9 ms, n=15) than that of WT (120.8 ± 7.0 ms, n=15) ($P < 0.05$). **(D-b)** APD₉₀ measured with the ruptured patch method. APD₉₀ of dnNRSF is 49.7 ± 2.8 ms (n=15); APD₉₀ of WT, 44.2 ± 4.9 ms (n=15). No statistical difference. **(E-a)** Duration of the AP measured at 0 mV (APD@0 mV) with the perforated patch method. APD@0 mV of dnNRSF was significantly shorter (1.87 ± 0.21 ms, n=15) than that of WT (6.35 ± 1.25 ms, n=15) ($P < 0.05$). **(E-b)** APD@0 mV measured with the ruptured patch method. dnNRSF, 1.74 ± 0.32 ms (n=15); WT, 5.16 ± 1.11 ms (n=15) ($P < 0.05$). dnNRSF, dominant negative mutant of the neuron restrictive silencing factor.

gation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Cell Isolation

Ventricular myocytes of dnNRSF mice and their WT littermates (14–16 weeks, body weight ~ 25 g) were obtained by a method reported previously.¹¹

Composition of the Solutions

For the perforated patch experiment, the pipette solution contained (mmol/L): 110 aspartic acid, 30 KCl, 5 K₂ATP, 5 Na₂ creatine phosphate, 0.1 Na₂GTP, 1 MgCl₂, 10 HEPES (pH=7.2 with KOH). Amphotericin (stock solution; 30 mg/ml in DEMSO) was added to this solution (final concentration:

0.3 mg/ml). For the ruptured whole-cell patch experiments, the low-Ca²⁺ pipette solution contained: 110 aspartic acid, 30 KCl, 5 K₂ATP, 5 Na₂ creatine phosphate, 0.1 Na₂GTP, 5 EGTA, 5 HEPES (pH=7.2 with KOH). To record the Ca²⁺ and Na⁺ currents, the electrode was filled with Cs⁺-rich pipette solution containing: 100 CsCl, 50 NMDG, 5 MgATP, 10 EGTA, 5 HEPES (pH=7.2 with CsOH). The physiological bathing solution contained: 140 NaCl, 5.4 KCl, 0.5 MgCl₂, 1.8 CaCl₂. To record Na⁺ and Ca²⁺ currents, KCl was replaced with CsCl. SEA0400 (Taisho Pharmaceutical) was dissolved in DEMSO as a stock solution (10 mmol/L).

Electrophysiology

AP and membrane current were recorded using an Axopatch200B amplifier and Digidata 1320 interface (Axon,

Figure 2. The reduction of I_{K1} density in dnNRSF myocytes. **(A-a)** The current traces of I_{K1} in a wild-type (WT) myocyte recorded in physiological bathing solution. **(A-b)** The family of current traces recorded in the presence of 1 mmol/L Ba^{2+} . The traces were recorded in the same myocyte as in **(A-a)**. **(B-a)** I_{K1} recorded in a dnNRSF myocyte. **(B-b)** In the same myocyte shown in **(B-a)**, 1 mmol/L Ba^{2+} completely inhibited I_{K1} , and disclosed the presence of I_h current at membrane potentials more negative than -80 mV. Note that the amplitude of the transient outward current at 0 mV is larger in dnNRSF. **(C)** Membrane capacitance of dnNRSF myocyte (181.1 ± 15.3 pF, $n=10$) was significantly larger than that of a WT myocyte (156.8 ± 10.2 pF, $n=10$) ($P<0.05$). **(D)** qPCR for Kir2.1 mRNA. Ratios of Kir2.1/GAPDH are indicated and are not statistically different between WT and dnNRSF myocytes. **(E-a)** The amplitudes of I_{K1} in a WT myocyte (black) and dnNRSF myocyte (red). The amplitude was measured at the ends of hyperpolarizing pulses. *Statistical difference ($n=10$, $P<0.05$). **(E-b)** The densities of I_{K1} in a WT myocyte (black) and dnNRSF myocyte (red). *Statistical difference ($n=10$, $P<0.05$). dnNRSF, dominant negative mutant of the neuron restrictive silencing factor; I_h , hyperpolarization-activated cation current; I_{K1} , inward rectifier K^+ current; qPCR, quantitative polymerase chain reaction.

CA, USA). The electrode resistance was 3–4 M Ω . All the experiments were carried out at 33–35°C.

Imaging

Myocytes were loaded with Indo1-AM (10 μ mol/L in the bathing solution) for 15 min at room temperature. Indo-1 was excited at 340–380 nm, and the fluorescent images were captured with a CCD camera. The intensity of fluorescence was measured at 405 $\pm$ 20 nm (F_{405}) and 480 $\pm$ 20 nm (F_{480}) using a beam-splitter and CCD camera (AquaCosmos Double View system, Hamamatsu photonics, Hamamatsu, Japan). Fluorescent images were continuously captured at the maximum rate available in our system (1 frame per 22 ms).

qPCR Analysis

Total RNA was obtained using Trizol reagent following manufacture's instruction. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using Platinum PCR SuperMix (Invitrogen, San Diego, CA, USA). Quantitative real-time RT-PCR (qPCR) was conducted for Kir2.1 and GAPDH using predesigned TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). The reaction was performed on an ABI Prism 7700 System

(Applied Biosystems). The mRNA levels of Kir2.1 were normalized to the endogenous GAPDH.

Statistical Analysis

Data are shown as mean $\pm$ SEM. Statistical difference was evaluated using Student's t-test. Differences were considered significant when $P<0.05$.

Results

AP Waveform of dnNRSF Cardiac Myocytes

We first recorded the APs of single ventricular myocytes under the condition of intact intracellular Ca^{2+} transient, because it has been reported that the specific blocker of the Na^+ - Ca^{2+} exchanger (NCX), SEA0400, suppresses the plateau phase of the AP in the mouse ventricle.¹² For this purpose, we used the perforated patch method. **Figure 1A** compares the AP waveform recorded in WT and dnNRSF myocytes; in the dnNRSF myocytes the resting membrane potential (RMP) was more depolarized, the AP duration (APD₉₀) was significantly prolonged, and early afterdepolarization (EAD) was observed in 37.3% (28 of 75 cells), as reported previously.¹⁰ As shown in **Figure 1A**, SEA0400

shortened the plateau phase in both WT and dnNRSF myocytes. Furthermore, SEA0400 completely suppressed EADs in dnNRSF myocytes. A similar effect has been reported in dogs.¹³ In contrast, when the AP was recorded using the ruptured patch method with low- Ca^{2+} pipette solution, the waveforms were almost superimposable in the presence and absence of SEA0400 (Figure 1B), presumably because the activity of the NCX was almost completely suppressed by chelating intracellular Ca^{2+} . Most notably, the RMP of dnNRSF myocytes was significantly more depolarized than that of WT myocytes, irrespective of the experimental conditions (Figure 1C). As summarized in Figure 1D, APD_{90} was significantly prolonged in the perforated patch experiments, whereas in the ruptured whole-cell patch experiments, APD_{90} was not statistically different between WT and dnNRSF myocytes. However, the rate of repolarization at -60 mV was significantly slower in dnNRSF myocyte, even in these experimental conditions ($1.05 \pm 0.11\text{ V/s}$ in WT ($n=15$); $0.62 \pm 0.08\text{ V/s}$ in dnNRSF ($n=15$); $P<0.05$). The APD measured at 0 mV ($\text{APD}@0\text{ mV}$) was, however, significantly shorter in dnNRSF myocytes in both experimental conditions (Figure 1E).

Reduction of the Inward-Rectifier K^+ Current Density

In ventricular myocytes, the RMP is primarily determined by the inward rectifier K^+ channel (I_{K1}), close to the equilibrium potential of K^+ (E_{K}). In order to explore the ionic mechanisms underlying the reduced RMP in dnNRSF myocytes, we first measured the amplitude of I_{K1} . In the experiments shown in Figures 2A,B, the membrane current was recorded using the ruptured whole-cell patch method and low- Ca^{2+} pipette solution. The family of current traces was activated by hyperpolarizing pulses from the holding potential of -40 mV , in the presence and absence of 1 mmol/L BaCl_2 . The amplitude of I_{K1} was defined as the Ba^{2+} -sensitive current. In dnNRSF myocytes, the hyperpolarization-activated cation current (I_{h}) was consistently disclosed in the presence of Ba^{2+} (Figure 2B-b). Upon depolarization to 0 mV , the amplitude of the transient outward current (I_{to}) appeared greater than that of the WT.

As is evident from the current-voltage (IV) relationship shown in Figure 2E-a, the amplitude of I_{K1} was significantly smaller in dnNRSF myocytes. Furthermore, the cellular capacitance was significantly larger in dnNRSF myocytes (Figure 2C). As a consequence, the density of I_{K1} was remarkably reduced in dnNRSF myocytes; the density of the inward I_{K1} at -140 mV was 56.0% and the density of the outward I_{K1} at -60 mV was 55.1% of WT myocytes, showing no voltage dependence in the inhibition (Figure 2E-b). The magnitude of the reduction of I_{K1} density appeared to be in good agreement with the reduction of dV/dt measured at -60 mV . In cardiac myocytes, Kir2.1 is a major molecular component of the I_{K1} channel, so we compared the amount of Kir2.1 mRNA using qPCR. However, there was no significant difference in the expression level of Kir2.1 (Figure 2D), which suggests that the reduction in I_{K1} density was caused by post-transcriptional mechanisms.

Transient Outward Current It has been reported that the voltage-gated outward currents, including I_{to} , play a major role in the modification of AP morphology in mouse cardiac myocytes.¹⁴ We compared the voltage-gated outward currents recorded in WT- and dnNRSF myocytes (Figure 3). In this experiment, the membrane current was activated from the holding potential of -90 mV in the physiological bathing solution. Therefore, robust activation of Na^+ current (I_{Na})

Figure 3. Comparison of the transient outward current (I_{to}) in wild-type (WT) and dnNRSF myocytes. (A) The family of current traces of I_{to} recorded in a WT myocyte. The pulse protocol is shown at the top. I_{to} was recorded in physiological bathing solution. The red dashed line indicates the zero current level. The robust inward current at the onset of depolarization is the voltage-gated Na^+ current (I_{Na}). The black circle indicates the position of the measuring the quasi-steady state amplitude of I_{to} . (B) Two components of I_{to} recorded in dnNRSF myocytes. The blue arrows indicate the rapidly inactivating I_{to} , which was additionally activated at membrane potentials more positive than -20 mV . The red circle indicates the same as in (A). (C) IV-relationship of quasi-steady state amplitude of I_{to} . The slowly inactivating I_{to} was activated at the membrane potentials more positive than -50 mV . WT, black symbols ($n=4$). dnNRSF, red symbols ($n=4$). The current density was not statistically different. dnNRSF, dominant negative mutant of the neuron restrictive silencing factor.

Figure 4. Na⁺-permeable, sustained inward current in dnNRSF myocytes. **(A)** Voltage-gated inward currents were activated by 2-step pulses. In the WT myocyte, no inward current was activated at -50 mV. I_{Ca-L} was activated at -10 mV. In the dnNRSF myocyte, late inward current was observed at the end of voltage stepping to -50 mV. When the Ca²⁺ concentration of the bathing solution was reduced from 1.8 mmol/L (black lines) to 0.1 mmol/L (red lines), the amplitude of the late inward current was increased (*). **(B)** Na⁺-sensitive nature of the late inward current activated at -50 mV. The current traces were recorded in the presence (black line) and absence (blue line) of 140 mmol/L Na⁺. The Ca²⁺ concentration was 1.8 mmol/L. The dashed line indicates the zero current level, and the red dotted line is the mean holding current at -90 mV. Note that late inward current was observed at -50 mV in the 140 mmol/L Na⁺ bathing solution. In Na⁺-free conditions, I_{Ca-T} was disclosed at the onset of the voltage stepping at -50 mV, whereas the late inward current was completely suppressed. **(C)** The late inward current activated by long voltage pulse (500 ms). The bathing solution contained 1.8 mmol/L Ca²⁺ and 140 mmol/L Na⁺. The dashed line and red dotted line indicate the same as in **(B)**. 10 μmol/L efendipine completely inhibited the late inward current. **(D)** IV-relationship of the late inward current. The current amplitudes were measured at the end of the 1st voltage pulse shown in the inset of **(A)**. Red symbols, 0.1 mmol/L Ca²⁺/140 mmol/L Na⁺. White symbols, 1.8 mmol/L Ca²⁺/0 mmol/L Na⁺ (n=8). **(E)** IV-relationship of I_{Ca-L} . Red symbols, dnNRSF (n=8); black symbols, WT (n=8). The peak amplitude of I_{Ca-L} was measured using the 2-step pulse protocol shown in **(A)**. dnNRSF, dominant negative mutant of the neuron restrictive silencing factor; I_{Ca-L} , L-type Ca²⁺ current; WT, wild-type.

and L-type Ca²⁺ current (I_{Ca-L}) overlapped during the activation phase of the outward currents, and interfered with the measurement of the peak amplitude of I_{Ca-L} . We therefore compared the amplitudes of quasi-steady state currents of I_{Ca-L} . The densities of the quasi-steady state currents were not statistically different at any of the membrane potentials examined (Figure 3B).

However, at the onset of depolarization pulses, rapidly inactivating I_{Ca-L} was observed in dnNRSF myocytes at membrane potentials more positive than -20 mV (Figure 3B). The molecular entities of this rapid I_{Ca-L} remain unclear at present. An outward tail current of I_{Ca-L} may be partly involved. Because the time course of inactivation was extremely rapid, this component seemed completely inactivated at the quasi-

Figure 5. Intracellular Ca^{2+} transient measured with Indo-1AM. The abscissa indicates the ratio of fluorescent intensity measured at 405 nm and 480 nm ($F_{405/480}$). The Indo-1AM loaded myocytes were perfused with physiological bathing solution kept at 33–35°C, and field-stimulated at 2 Hz. **(A)** Continuous trace of $F_{405/480}$ recorded in WT myocytes. The application of 0.3 $\mu\text{mol/L}$ isoproterenol is indicated by the white bar. The inset shows the expanded traces of $F_{405/480}$ for 3 s, in the presence and absence of isoproterenol. The dashed line indicates $F_{405/480}=0.25$. **(B)** $F_{405/480}$ trace measured in dnNRSF myocytes. After the application of 0.3 $\mu\text{mol/L}$ isoproterenol, diastolic $F_{405/480}$ slightly increased, followed by spontaneous oscillation. The inset shows the expanded trace at the onset of spontaneous oscillation for 3 s. The dashed line indicates $F_{405/480}=0.25$. **(C)** Summary of $F_{405/480}$ measured in WT and dnNRSF cardiac myocytes ($n=15$, $P<0.05$). The black bars indicate diastolic $F_{405/480}$, and the red bars, systolic $F_{405/480}$. In both the presence and absence of isoproterenol, diastolic $F_{405/480}$ was significantly higher in dnNRSF ($n=15$, $P<0.05$). dnNRSF, dominant negative mutant of the neuron restrictive silencing factor; WT, wild-type.

steady state in both WT and dnNRSF (Figures 3A,B). As shown in Figure 1, ADP@0mV was shorter in dnNRSF myocytes, presumably because the amplitude of the rapid I_{to} was larger in dnNRSF myocytes.

Na⁺-Permeable Late Current It has been reported that the late Na^+ current is increased in myocytes from a heart failure model.^{15,16} Therefore, we explored the possibility that Na^+ conductance might contribute to the reduced RMP in dnNRSF. In the experiments shown in Figure 4A, we activated the voltage-gated I_{Na} and $I_{\text{Ca-L}}$ using 2-step voltage pulses. The K^+ conductance were completely suppressed by using a Cs^+ -rich pipette solution and by replacing extracellular K^+ with Cs^+ . In 8 of 40 myocytes isolated from dnNRSF,

a sustained inward component was observed at the end of voltage stepping to -50mV . When the extracellular Ca^{2+} concentration was reduced from 1.8 mmol/L to 0.1 mmol/L, the sustained inward component at -50mV was clearly enlarged. However, such a sustained inward component was never recorded in WT myocytes.

In order to clarify the ionic selectivity of this component, dnNRSF myocytes were perfused with Na^+ -free, 1.8 mmol/L Ca^{2+} solution. As shown in Figure 4B, the inward current jump at -50mV in 140 mmol/L Na^+ solution was completely suppressed, and activation of the $I_{\text{Ca-T}}$ followed by an outward current jump was disclosed. $I_{\text{Ca-T}}$ was recorded in a small population of dnNRSF myocytes (5 of 75). Unexpected-

edly, we found that this inward current jump at -50 mV was sensitive to calcium-channel blockers. As shown in **Figure 4C**, $10\text{ }\mu\text{mol/L}$ efonidipine almost completely inhibited the Na^+ -permeable, sustained inward current (I_{st}) at -50 mV. **Figure 4D** shows the IV relationship of I_{st} measured at the end of the depolarizing pulse in $140\text{ mmol/L Na}^+/0.1\text{ mmol/L Ca}^{2+}$ solution, and in Na^+ -free/ $0.1\text{ mmol/L Ca}^{2+}$ solution. I_{st} was activated at membrane potentials more positive than -60 mV, and the reversal potential was between 10 and 20 mV.

The amplitude of $I_{\text{Ca-L}}$ also affects the morphology of the AP of cardiac myocytes. We therefore compared the amplitude of $I_{\text{Ca-L}}$ activated by a 2-step pulse protocol (**Figure 4A**). As is evident from the IV-relationship shown in **Figure 4E**, the density of $I_{\text{Ca-L}}$ was not significantly different between WT and dnNRSF myocytes at any of the membrane potentials examined. However, the profile of the IV-relationship was slightly different: in WT myocytes, the peak of the IV relationship was at -10 mV, whereas in dnNRSF myocytes, it was at -20 mV.

Intracellular Ca^{2+} Transient

Alteration of intracellular Ca^{2+} handling as part of the remodeling in the hypertrophied or failing heart is well known.¹⁷ As shown in **Figure 1**, the EAD and prolonged APD observed in dnNRSF myocytes were completely suppressed by a specific blocker of the NCX. Therefore, it appears reasonable to expect that intracellular Ca^{2+} handling is also altered in dnNRSF myocytes. We compared the intracellular Ca^{2+} transient of intact myocytes isolated from WT and dnNRSF (**Figure 5**). The myocytes were loaded with Indo-1-AM, and the ratio of fluorescent intensity was measured at 405 nm and 480 nm ($F_{405/480}$). The Ca^{2+} transient of myocytes was induced by field stimulation at 2 Hz . As summarized in **Figure 5C**, diastolic $F_{405/480}$ was 0.22 ± 0.02 in WT myocytes ($n=15$). In dnNRSF myocytes, the diastolic intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was significantly higher; $F_{405/480}$ was 0.24 ± 0.02 ($n=15$) ($P<0.05$). In contrast, systolic $F_{405/480}$ was not significantly different between WT (0.28 ± 0.02 , $n=15$) and dnNRSF myocytes (0.29 ± 0.02 , $n=15$).

Adrenergic stimulation seemed to trigger abnormal electrical activity in dnNRSF myocytes, as reported previously.¹⁰ We therefore compared the effects of $0.3\text{ }\mu\text{mol/L}$ isoproterenol on the Ca^{2+} transient. As shown in **Figure 5A**, the peak $[\text{Ca}^{2+}]_i$ of WT myocytes increased promptly after application of isoproterenol, and reached a steady level within approximately 40 s . The diastolic $[\text{Ca}^{2+}]_i$ decreased slightly but transiently from the control level. In dnNRSF myocytes, the systolic $[\text{Ca}^{2+}]_i$ was also increased by isoproterenol, demonstrating that the response to β -adrenergic stimulation was retained. However, diastolic $[\text{Ca}^{2+}]_i$ slowly increased after the application of isoproterenol and spontaneous $[\text{Ca}^{2+}]_i$ oscillation was induced (**Figure 5B inset**). Spontaneous $[\text{Ca}^{2+}]_i$ oscillation presumably activates the Ca^{2+} -dependent inward current carried by the NCX, giving rise to the abnormal electrical activity of dnNRSF myocytes.¹⁰

In our experimental system, the maximal rate of image capture was 1 frame per 22 ms . Because of this limitation, the recording of the Ca^{2+} transient was discontinuous; we could obtain only 22 data points with 22-ms interval in 1 cycle (500 ms) of field stimulation. Therefore, it was practically difficult to evaluate the precise values of the time-to-peak and the half relaxation time of the $[\text{Ca}^{2+}]_i$ transient during the AP of mouse cardiac myocytes.

Discussion

In animal models of the hypertrophied or failing heart, physiological remodeling so far reported may be summarized as follows. First, alteration of intracellular Ca^{2+} handling, such as Ca^{2+} leak from the sarcoplasmic reticulum and downregulation of Ca^{2+} -ATPase. Second, downregulation of the “repolarization reserve”, such as decreased outward currents and/or increased inward currents. Third, re-expression of fetal-type genes, such as contractile proteins and ion channels.^{1–4} Of these physiological remodeling processes, it has been suggested that transcriptional repressor REST/NRSF primarily plays a role in the regulation of fetal cardiac genes. In the heart, REST/NRSF represses the transcription of fetal cardiac genes in mature animals, and the hypertrophic stimulation appears to release, at least in part, the transcriptional repression by REST/NRSF.¹⁸

In the present study, we have demonstrated that inhibition of the REST/NRSF function significantly downregulated the “repolarization reserve” of ventricular myocytes, in addition to the re-expression of fetal-type ion channels such as I_{h} and $I_{\text{Ca-T}}$. The density of I_{K1} was remarkably reduced, in spite of the mRNA level of Kir2.1 being unchanged. The mechanisms underlying the reduction in I_{K1} density is not clear at present. It has been reported that elevation of diastolic $[\text{Ca}^{2+}]_i$ reduces I_{K1} density in rat hypertrophied myocytes, via protein kinase C (PKC)-dependent and PKC-independent pathways.¹⁹ Therefore, it might be speculated that in the hypertrophied heart a series of physiological changes (eg, reduction of I_{K1} density, depolarization of RMP, reduction of the driving force of NCX, elevation of diastolic $[\text{Ca}^{2+}]_i$) could form a vicious spiral. Because diastolic $[\text{Ca}^{2+}]_i$ was also elevated in the dnNRSF myocytes (**Figure 4**), similar mechanisms might be involved in the reduction of I_{K1} density.

I_{to} is an important determinant of AP morphology in mouse cardiac myocytes, and a decrease in the I_{to} density should also reduce the “repolarization reserve”.¹⁴ In the present study, the density of the slow component of I_{to} was not decreased in dnNRSF myocytes. In contrast, the density of rapidly inactivating I_{to} was increased. Because rapid I_{to} was only activated at membrane potentials $>-20\text{ mV}$, and was completely inactivated within 5 ms , this component might modify only the duration of overshoot, without affecting the duration of the plateau phase at -40 mV . Vice versa, the activation of rapid I_{to} might increase the driving force of $I_{\text{Ca-L}}$, giving rise to a transient increase of Ca^{2+} influx.

As a mechanism of the reduction of the “repolarization reserve” and the generation of EAD in the hypertrophied heart, the late I_{Na} is now receiving much attention.^{15,16} The late I_{Na} (ie, delayed inactivation of I_{Na}) seems to be regulated by calmodulin kinase II (CaMKII).²⁰ In dnNRSF myocytes, it appears reasonable to expect that I_{Na} was modified by CaMKII, which was activated by the elevation of diastolic $[\text{Ca}^{2+}]_i$. In fact, the Na^+ -permeable, sustained I_{st} was observed in a small population of dnNRSF myocytes. Increase of Na^+ influx through I_{st} and I_{h} is anticipated in dnNRSF myocytes. Alteration of Na^+ homeostasis may reduce of the driving force of the NCX, thereby increasing $[\text{Ca}^{2+}]_i$.²¹

It has been reported that late I_{Na} is sensitive to ranolazine.¹⁶ Although we have not yet examined the effect of ranolazine on I_{st} in dnNRSF myocytes, this current might not be delayed inactivation of I_{Na} , because I_{st} in dnNRSF myocytes was sensitive to calcium-channel blocker (**Figure 3C**), showing a similarity to the sustained inward current reported in the sino-atrial node.²² We have previously reported that

efonidipine rescued the lethal arrhythmia of dnNRSF mice.¹⁰ Therefore, in addition to I_{Ca-L} and I_{Ca-T} , I_{st} might be an important pharmacological target in dnNRSF myocytes.

Study Limitations

It still remains unclear which of the primary genetic alternations caused by dnNRSF initiates the physiological remodeling series. Because diastolic $[Ca^{2+}]_i$ was elevated in dnNRSF myocytes, Ca^{2+} -dependent genetic alterations might have subsequently occurred.²³ It has been recently reported that Cav3.2 (*Cacna1h*) is required for cardiac hypertrophy induced by pressure overload.²⁴ Likewise, crossbreeding of dnNRSF mice with transgenic mice lacking the target genes of REST/NRSF might provide a clue.

Conclusion

We found that RMP was reduced, and APD₉₀ prolonged in dnNRSF myocytes. These changes were caused by a reduction in the “repolarization reserve”, which in turn was caused by reduced I_{K1} density and the emergence of a Na^+ -permeable, late inward current. The diastolic $[Ca^{2+}]_i$ was elevated, and spontaneous oscillation of $[Ca^{2+}]_i$ was induced by β -adrenergic stimulation. Under these conditions, the amplitude of EAD generated by the inward NCX current might be increased, thereby increasing the vulnerability to ventricular arrhythmia.

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