Post-transcriptional silencing of SCN1B and SCN2B genes modulates late sodium current in cardiac myocytes from normal dogs and dogs with chronic heart failure

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Mishra S, Undrovinas NA, Maltsev VA, Reznikov V, Sabbah HN, Undrovinas A. Post-transcriptional silencing of SCN1B and SCN2B genes modulates late sodium current in cardiac myocytes from normal dogs and dogs with chronic heart failure. Am J Physiol Heart Circ Physiol 301: H1596–H1605, 2011. First published June 24, 2011; doi:10.1152/ajpheart.00948.2009.—The emerging paradigm for Na+ current in chronic heart failure (HF) is that its transient component (I\text{Na$_T$}) responsible for the action potential (AP) upstroke is decreased, whereas the late component (I\text{Na$_L$}) involved in AP plateau is augmented. Here we tested whether Na\text{\beta$_1$}- and Na\text{\beta$_2$}-subunits can modulate I\text{Na$_L$} parameters in normal and failing ventricular cardiomyocytes (VCMs). Chronic HF was produced in nine dogs by multiple sequential coronary artery microembolizations, and six dogs served as a control. I\text{Na$_L$} and APs were measured by the whole cell and perforated patch-clamp in freshly isolated and cultured VCMs, respectively. I\text{Na$_L$} was augmented with slower decay in HF VCMs compared with normal heart VCMs, and these properties remained unchanged within 5 days of culture. Post-transcriptional silencing SCN1B and SCN2B were achieved by virally delivered short interfering RNA (siRNA) specific to Na\text{\beta$_1$} and Na\text{\beta$_2$}. The delivery and efficiency of siRNA were evaluated by green fluorescent protein expression, by the real-time RT-PCR, and Western blots, respectively. Five days after infection, the levels of mRNA and protein for Na\text{\beta$_1$} and Na\text{\beta$_2$} were reduced by >80%, but mRNA and protein of Na\text{\beta$_{1.5}$}, as well as I\text{Na$_T$}, remained unchanged in HF VCMs. Na\text{\beta$_1$}-siRNA reduced I\text{Na$_L$} density and accelerated I\text{Na$_L$}, two-exponential decay, whereas Na\text{\beta$_2$}-siRNA produced an opposite effect in VCMs from both normal and failing hearts. Physiological importance of the discovered I\text{Na$_L$} modulation to affect AP shape and duration was illustrated both experimentally and by numerical simulations of a VCM excitation-contraction coupling model. We conclude that in myocytes of normal and failing dog hearts Na\text{\beta$_1$} and Na\text{\beta$_2$} exhibit oppositely directed modulation of I\text{Na$_L$}.

THE EMERGING PARADIGM FOR Na+ CURRENT (I\text{Na$_L$}) IN CHRONIC HEART FAILURE (HF) IS THAT ITS TRANSIENT COMPONENT (I\text{Na$_T$}) RESPONSIBLE FOR THE ACTION POTENTIAL (AP) UPSTROKE AND EXCITATION PROPAGATION IS DECREASED, WHEREAS THE LATE COMPONENT (I\text{Na$_L$}) INVOLVED IN AP PLATEAU IS AUGMENTED (21, 23, 25, 42, 45, 48). MOLECULAR MECHANISMS OF THESE HF-RELATED I\text{Na$_L$} ALTERATIONS ARE STILL UNDERSTUDIED. NUMEROUS STUDIES INDICATE A POSSIBLE ROLE OF Na\text{\beta} AUXILIARY SUBUNITS TO MODULATE Na+ CHANNEL (NaCh) EXPRESSION AND FUNCTION (27), BUT IMPLICATIONS OF Na\text{\beta} IN I\text{Na$_L$} MODULATION HAVE NOT BEEN STUDIED IN DETAIL, ESPECIALLY IN HF. OUR PREVIOUS STUDIES IN A CANINE CHRONIC HF MODEL SHOVED THAT THE PROTEIN LEVEL OF THE MAIN NaCh ISOFORM EXPRESSED IN THE HEART, Na\text{\beta$_{1.5}$}, UNDERLYING I\text{Na$_L$} (18), IS REDUCED BUT REMAINS UNCHANGED FOR Na\text{\beta$_1$}- AND Na\text{\beta$_2$}-SUBUNITS, MAKING THESE β-SUBUNITS RELATIVELY UPREGULATED (48). ANS AN INTRIGUING POSSIBILITY COULD BE THAT DIFFERENTIAL EXPRESSION OF Na\text{\beta$_{1.5}$} AND Na\text{\beta$_2$}-SUBUNITS IN NORMAL AND FAILING HEARTS CAN CONtribute, AT LEAST IN PART, TO I\text{Na$_L$} ALTERATIONS OBSERVED IN HF.

IN THE PRESENT STUDY USING A COMBINATION OF EXPERIMENTAL AND NUMERICAL MODELING APPROACHES, WE TESTED A HYPOTHESIS THAT Na\text{\beta$_1$} AND Na\text{\beta$_2$} CAN MODULATE I\text{Na$_L$} IN VENTRICULAR CARDIOMYOCYTES (VCMs) ISOLATED FROM ADULT FAILING DOG HEARTS. WE PERFORMED POST-TRANSCRIPTIONAL SILENCING OF SCN1B AND SCN2B GENES BY THE SEQUENCE-DIRECTED RNA INTERFERENCE USING SHORT INTERFERING RNA (siRNA). WE TOOK ADVANTAGE OF THE ESTABLISHED ADULT DOG VCMs CULTURE MODEL THAT PRESERVES I\text{Na$_L$} AND I\text{Na$_T$} OVER 5 DAYS, I.E., SUFFICIENT TIME FOR GENE SILENCING AND MEMBRANE PROTEIN TURNOVER (18). WE FOUND THAT SILENCING OF SCN1B AND SCN2B GENES SIGNIFICANTLY LESSENS OR ENHANCES I\text{Na$_L$}, RESPECTIVELY. PHYSIOLOGICAL SIGNIFICANCE OF THESE β-SUBUNITS–RELATED MODULATIONS OF I\text{Na$_L$} WAS ILLUSTRATED BY THE CONCOMITANT AP CHANGES BOTH EXPERIMENTALLY AND IN SILICO.

MATERIALS AND METHODS

HF MODEL

THE STUDY CONFORMS TO THE GUIDELINES FOR CARE AND USE OF LABORATORY ANIMALS PUBLISHED BY THE NATIONAL INSTITUTES OF HEALTH AND WAS APPROVED BY THE ANIMAL CARE AND USE COMMITTEE OF THE HENRY FORD HEALTH SYSTEM. CHRONIC HF THAT IS SIMILAR BY A VAST ARRAY OF FUNCTIONAL AND PATHOPHYSIOLOGICAL PARAMETERS (35) TO THAT IN HUMANS WAS PRODUCED IN NINE DOGS BY MULTIPLE SEQUENTIAL CORONARY ARTERY MICROSPHERE EMBOLIZATIONS AS PREVIOUSLY DESCRIBED (36). SIX NORMAL DOGS SERVE AS A CONTROL. AT THE TIME OF HARVESTING THE HEART (~3 mo AFTER LAST EMBOLIZATION), LEFT VENTRICULAR (LV) EJECTION FRACTION WAS ~25%.

CELL CULTURE AND TRANSFECTION

Midmyocardial VCMs were enzymatically isolated from the apical LV midmyocardial slices as reported previously (21). The yield of viable rod-shaped, Ca2+-tolerant VCMs varied from 40% to 70%. VCMs were cultured for 5 days [the time frame required for the gene silencing (18)], as described previously (23). During this time, VCMs manifested slightly rounded edges, i.e., well-known characteristics for the cultured adult cardiomyocytes (9, 31). Adenovirus-delivered siRNAs did not further affect morphol-
ogy of VCMs (Supplemental Fig. S1). We added 10 μl of 5 × 10^8 virus particle/ml directly to the medium of culture dish containing ∼15 × 10^4 cells and cultured them further during the next 4 days (5 days total). Effectiveness of transfection was visually monitored in individual cardiomyocytes (Nikon Diaphot 200) by green fluorescent protein (GFP) fluorescence (Fig. 2A, inset, and Supplemental Fig. S1).

**siRNA Design**

Double-stranded hairpin siRNAs corresponding to the previously sequenced dog Na,β_1_ and Na,β_2_ (GenBank DQ061859 and AY263393, respectively) were designed as recommended (8). The details and sequences of silencing and nonsilencing control siRNAs used in this study are given in Supplemental Table S1. A Blast search for these sequences against the GenBank database was performed and did not reveal matches with any other gene.

**Real-time PCR and Western Blot Analysis**

To quantify mRNA levels, we used the fluorescence-based (cyan green) kinetic real-time PCR performed using 7500 Fast Real-Time PCR System Applied Biosystems sequence detection system. The relative mRNA abundance expressed as arbitrary units was calculated using the expression levels of all transcripts normalized to GAPDH mRNA. This value was then normalized to mRNA levels measured from VCMs transfected with the nonsilencing siRNA for the appropriate transcript (2−ΔΔCt method). The sets of primers are given in Supplemental Table S2. Membrane protein preparations were obtained from ∼3 × 10^6 myocytes as previously described (Ref. 48; Supplemental Material for details). Antibody was detected with Western blot chemoluminescence reagent (NEN Life Science Product). Western blots were considered specific if the peptide epitope reduced the band intensity. The resulting images of Western blots were
scanned, and relative densities of bands were quantified using Sigma-Gel or ImageJ software, which includes a background subtraction algorithm. The primary polyclonal anti-Na,β1 and anti-Na,β2 antibodies were obtained from Cell Applications (San Diego, CA) and Alomone Labs (Jerusalem, Israel), respectively. Representative full Western blots with these antibodies are shown in the Supplemental Fig. S2. Polyclonal calsequestrin antibody (Abcam) was used for the protein loading control.

Fig. 2. Post-transcriptional silencing of Na,β1-or Na,β2-subunits expression does not affect the whole cell transient sodium current, $I_{NaT}$, in patch-clamped cultured left ventricular cardiac myocytes from dogs with chronic HF. A: representative raw traces of $I_{NaT}$ were recorded at different membrane potentials ($V_m$) in cells 5 days after infection with the virus containing control nonsilencing siRNA (control, left), β1-siRNA (middle), or β2-siRNA (right). Image of cells expressing green fluorescent protein (GFP) is shown at left inset. B: average data for peak $I_{NaT}$-voltage relationship obtained in cultured cells for 5 days with virally delivered control siRNA, Na,β1-siRNA, and Na,β2-siRNA. Solid lines show theoretical curves fit to current-voltage ($I$-$V$) to evaluate steady-state activation (SSA) parameters (Supplemental Eq. S2, solid dashed and dotted lines, respectively). C: average experimental data of steady-state inactivation (SSI) data points along with the fit to a Boltzmann function (Supplemental Eq. S3, solid, dashed, and dotted lines, respectively). No statistically significant difference was found when theoretical SSI curves were compared ($F$ test). D: decay time constants of $I_{NaT}$ (double exponential fit; Supplemental Eq. S1) were evaluated at the different membrane potentials. E: maximum density of $I_{NaT}$ remained unchanged in these conditions. Data in B–D represents means ± SE and were pooled from 5 to 8 cells. Detailed statistics for all SSA and SSI parameters of the theoretical fits shown in B and C are presented in Table 1. Voltage-clamp protocols are shown in B and C, insets. $V_h$, holding potential; $V_p$, prepulse.
Electrophysiology

$I_{Na}$ and AP were recorded using a conventional whole cell and perforated patch-clamp techniques, respectively. The details of a double exponential fit to decay kinetics of the $I_{Na,t}$ and $I_{Na,f}$, as well as evaluation of the steady state activation (SSA) and steady state inactivation (SSI) voltage dependency is given in the Supplemental Material (Supplemental Equations S1, S2, and S3, respectively).

Numerical AP Model

To address physiological significance of $I_{Na,t}$ modulation by β-subunits, we used a modified excitation-contraction (E-C) coupling model of normal and failing canine ventricular myocyte developed previously by Winslow et al. (46). Our model modification to include $I_{Na,t}$ equations has been recently published (44).

All experimental procedures and numerical modeling details (including numerical values of parameters used in the present study) are available in the Supplemental Material (expanded Supplemental Methods section).

Statistical Analysis

Statistics are reported as means ± SE with n representing the number of cells. Multiple comparisons between treatment groups were made using one-way ANOVA followed by the Bonferroni’s post hoc test. The significance of changes of theoretical SSA and SSI parameters for $I_{Na,t}$ were made using one-way ANOVA followed by the Bonferroni’s post hoc test. Multiple comparisons between treatment groups were considered statistically significant for $P < 0.05$.

RESULTS

Efficacy of siRNAs to Silence SCN1B and SCN2B Genes

The efficacy of virally delivered siRNAs to silence concomitant SCN1B and SCN2B genes in cultured VCMs was assessed by changes in both mRNA transcript levels and membrane protein levels of Na$_\alpha$1.5, or other auxiliary β-subunits, not targeted by the siRNA, remained unchanged in these cells (Fig. 1A). Parallel to mRNA, membrane protein levels for both Na$_\alpha$1.5 (Fig. 1C) and Na$_\alpha$2.2 (Fig. 1D) were also dramatically reduced in response to the siRNA treatment. These results confirm efficacy and specificity of the virally delivered siRNAs to silence SCN1B and SCN2B genes in cultured adult dog VCMs.

Post-transcriptional Silencing of SCN1B and/or SCN2B Genes Does Not Affect $I_{Na,t}$

We tested whether reduced expression of Na$_\alpha$1.5 or Na$_\alpha$2.2 can affect $I_{Na,t}$. Figure 2A shows representative family of current traces at different depolarization steps to evaluate the current voltage ($I-V$) relationship in cells transfected with control nonsilencing siRNA (Fig. 2, left) and with siRNAs designed to silence either SCN1B (Fig. 2, middle) or SCN2B (Fig. 2, right) genes. We found that $I-V$, SSA (Fig. 2B), SSI, (Fig. 2C), two-exponential decay kinetics (Fig. 2D), and density for $I_{Na,t}$ (Fig. 2E) remained unchanged in VCMs with silenced SCN1B or SCN2B genes, respectively. Statistical data for SSA and SSI parameters for these experiments are given in Table 1.

Modulation of $I_{Na,t}$ by Na$_\alpha$1.5 and Na$_\alpha$2.2 Subunits

At first we tested whether primary cell culture affects the established paradigm that $I_{Na,t}$ is augmented and slower in failing compared with normal hearts (25, 42, 45). It is evident

Table 1. Voltage dependence of SSA and SSI for $I_{Na}$ in ventricular cardiomyocytes from normal dogs and dogs with chronic heart failure

<table>
<thead>
<tr>
<th>Conditions</th>
<th>SSA Parameters</th>
<th>SSI Parameters</th>
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<tbody>
<tr>
<td></td>
<td>$V_{1/2A}$, mV</td>
<td>$k_A$, mV</td>
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<tr>
<td>Normal heart, $I_{Na,t}$</td>
<td></td>
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<tr>
<td>Control siRNA</td>
<td>−82.0 ± 0.6</td>
<td>−5.9 ± 0.8</td>
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<tr>
<td>Na$_\alpha$1.5-siRNA</td>
<td>−81.9 ± 0.7</td>
<td>−6.2 ± 0.6</td>
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<tr>
<td>Na$_\alpha$2.2-siRNA</td>
<td>−82.1 ± 1.8</td>
<td>−6.1 ± 1.1</td>
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<tr>
<td>Failing heart, $I_{Na,f}$</td>
<td></td>
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<tr>
<td>Control siRNA</td>
<td>−81.6 ± 1.7</td>
<td>−6.7 ± 0.7</td>
</tr>
<tr>
<td>Na$_\alpha$1.5-siRNA</td>
<td>−82.2 ± 1.4</td>
<td>−5.9 ± 0.9</td>
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<tr>
<td>Na$_\alpha$2.2-siRNA</td>
<td>−81.9 ± 1.5</td>
<td>−6.4 ± 0.7</td>
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<tr>
<td>Normal heart, $I_{Na,t}$</td>
<td></td>
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<tr>
<td>Control siRNA</td>
<td>−81.7 ± 0.5</td>
<td>−6.2 ± 0.5</td>
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<tr>
<td>Na$_\alpha$1.5-siRNA</td>
<td>−80.2 ± 1.6</td>
<td>−6.4 ± 0.4</td>
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<tr>
<td>Na$_\alpha$2.2-siRNA</td>
<td>−80.9 ± 1.4</td>
<td>−6.2 ± 0.8</td>
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<tr>
<td>Failing heart, $I_{Na,f}$</td>
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<tr>
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<tr>
<td>Na$_\alpha$2.2-siRNA</td>
<td>−81.6 ± 1.2</td>
<td>−6.6 ± 0.4</td>
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Values are means ± SE; n, cell number. Steady-state inactivation and steady-state activation (SSI and SSA) parameters were obtained from Na$^+$ current ($I_{Na,t}$) data fit to the Supplemental Eqs. S2 and S3, respectively. We did not find statistically significant differences between parameters within the experimental groups evaluated by the ANOVA followed by Bonferroni’s post hoc test. siRNA, short interfering RNA; $I_{Na,t}$, transient Na$^+$ component; $I_{Na,f}$, late Na$^+$ component; $V_{1/2A}$, and $k_A$, midpoint and the slope of the respective Boltzmann function underlying the NaCh SSA; $V_{1/2S}$ an $k_S$, midpoint and slope of the relationship of SSI parameters.
from Fig. 3, B–D, that within 5 days culture $I_{\text{NaL}}$ remained robust in both VCMs from normal and failing hearts, and the paradigm was preserved. Moreover, our infection procedure did not alter $I_{\text{NaL}}$ parameters in failing VCMs when freshly isolated and treated with virus containing nonsilencing siRNA were compared (Fig. 3, B–D). In contrast with $I_{\text{NaT}}$, we found a significant but opposite change in $I_{\text{NaL}}$ in response to reduced expression of Na$_x$ß$_1$ or Na$_x$ß$_2$ by the siRNAs (Fig. 3). As evident from the figure, in VCMs isolated from both normal and failing hearts, reduction of Na$_x$ß$_1$ expression resulted in $I_{\text{NaL}}$ decay acceleration and density reduction. In contrast, $I_{\text{NaL}}$ decay was delayed and density was increased in VCMs with reduced levels of Na$_x$ß$_2$. Figure 4A shows $I-V$ relationship for these experiments. Clearly, reduction of Na$_x$ß$_1$ or Na$_x$ß$_2$ expression reduced or increased density of $I_{\text{NaL}}$ within the wide range of membrane potentials including potentials corresponding to the AP plateau, respectively. SSA (Fig. 4A) and SSI (Fig. 4B) parameters remained unchanged in these conditions (statistical data are given in Table 1).

Na$_x$ß$_1$ and Na$_x$ß$_2$ Subunits Modulate AP Duration

We recorded APs in response to different stimulation rates (0.25, 0.5, and 1.0 Hz) in VCMs treated with control nonsilencing siRNA and with siRNAs directed to silence either SCN1B or SCN2B genes. Figure 5A shows representative traces of APs recorded in these conditions. Statistical data on APD modulation by SCN1B and SCN2B genes is shown in Fig. 5B. Reduced expression of Na$_x$ß$_1$ caused significant shortening of APD at all pacing rates tested. In contrast, reduction of Na$_x$ß$_2$ expression significantly in-
increased APD. Because ion currents change during primary culture of adult VCMs (30, 31) (see Study Limitations for inherent constraints of electrophysiological studies of the cultured VCMs), the effect of \( I_{\text{NaL}} \) modulation by the β-subunits on APs of the ventricular myocytes of the failing heart needs to be further evaluated. For this purpose we used E-C coupling model of canine ventricular myocytes (46) with new formulations of \( I_{\text{NaL}} \) (44), in which we included experimentally measured \( I_{\text{NaL}} \) modulation by β-subunits (the model details are given in the Supplement Material). Figure 6 shows simulated AP traces and simultaneous \( I_{\text{NaL}} \) traces in control and after silencing of Na\(_{\beta 1}\) or Na\(_{\beta 2}\) subunits in VCMs of both normal and failing hearts at a stimulation rate of 1.0 Hz, respectively. Simulated APs become notably shortened or prolonged in response to \( I_{\text{NaL}} \) changes caused by the reduction of the Na\(_{\beta 1}\) or Na\(_{\beta 2}\) subunit expression, respectively.

**DISCUSSION**

**Result Summary and Possible Importance**

For the first time, we report a highly effective RNA interference-based method for post-transcriptional silencing SCN1B and/or SCN2B genes in cultured adult cardiac myocytes isolated from the left ventricles of normal dogs and dogs with chronic HF. The Na\(_{\beta 1}\)-siRNA and Na\(_{\beta 2}\)-siRNA used in this study were virally delivered and caused ~80% reduction of expression of SCN1B and/or SCN2B genes assessed by the mRNA transcript levels and the membrane protein content. The whole cell transient current, \( I_{\text{NaL}} \), was not affected by the reduced expression of either Na\(_{\beta 1}\) or Na\(_{\beta 2}\) but caused a significant loss-of-function or gain-of-function of \( I_{\text{NaL}} \), respectively. Post-transcriptional SCN1B and/or SCN2B gene silencing effects on \( I_{\text{NaL}} \) were similar for normal and failing hearts. Our experiments and in silico simulations predict notable AP duration changes within modulation range of \( I_{\text{NaL}} \) by both β-subunits measured experimentally, thus indicating that this modulation could be a physiologically important mechanism of AP regulation in both normal and failing myocardium (Figs. 5 and 6). We also simulated \( I_{\text{NaL}} \) dynamics underlying the AP changes (Fig. 6, C and D). Please note that simulated \( I_{\text{NaL}} \) profile during APs well corresponds to the respective experimentally measured \( I_{\text{NaL}} \) profile reported previously in canine VCMs (3).

Many prior studies have demonstrated an important role of \( I_{\text{NaL}} \). It was shown that \( I_{\text{NaL}} \) reduction by 50–60% by tetrodotoxin (TTX), saxitoxin, counter-current injection, or a specific \( I_{\text{NaL}} \) blocker ranolazine, i.e., in the range similar to Na\(_{\beta 1}\)-siRNA-related modulation (Figs. 3 and 4), causes significant physiological effects in freshly isolated human and dog HF VCMs (21, 41, 42, 44), specifically 1) reduced AP duration and duration variability, 2) eliminated EADs, 3) improved single cell contractility, and 4) reduced diastolic Ca\(^{2+}\) accumulation. Therefore, in this regard overall \( I_{\text{NaL}} \) decrease produced by silencing SCN1B reported in the present study is expected to be physiological significant. However, silencing SCN2B gene causes overall \( I_{\text{NaL}} \) increase, i.e., similar to that reported for HF (Figs. 3, 4, and 5; Refs. 41, 44, and 45). Therefore, targeting SCN1B but not SCN2B gene with the aim to modulate \( I_{\text{NaL}} \) in HF is likely to be considered as a plausible target for the future gene therapy.

**Cultured Adult Cardiomyocytes as a Model to Study NaCh Regulation**

Primary cultures of cardiomyocytes are a useful model for broad reasons: 1) it produces a substantial amount of viable homogeneous population of terminally differentiated cells free of extracellular matrix and neurohumoral factors and...
compared with control. Sipperly systems and, recently, in the native heart tissues although extensively studied in the past both in the heterologous expres-

Indeed the primary VCMs culture. Indeed it has been shown that low doses of TTX, which preferentially blocks neuronal NaCh, reduce AP duration in cardiac Purkinje fibers (6) and block the persistent non-inactivating INaL current in the rat (13, 37). Single channel studies revealed multiple conductance levels of NaCh also pointing to a possible contribution of the different (neuronal) NaCh isoforms to the INaL component in the native NaCh environment of VCMs isolated from both normal and failing hearts. Below we discuss three important questions of the molecular mechanisms of INaL modulation by the β-subunits.

Which Naβ underlies INaL in the heart and, therefore, is targeted by the SCN1B and SCN2B silencing? Using antisense morpholino oligonucleotides we have recently reported that Naβ1.5 is the major (at least 60%) contributor to the INaL in adult cardiac myocytes from normal dogs (18). Although the major target in our study is likely Naβ1.5, the neuronal NaCh isoforms (Naβ1.1, Naβ1.2, Naβ1.3) reported in the heart (11, 15, 16) may also contribute to INaL. Indeed it has been shown that low doses of TTX, which preferentially blocks neuronal NaCh, reduce AP duration in cardiac Purkinje fibers (6) and block the persistent non-inactivating INaL current in the rat (13, 37).

2) It is suitable for patch-clamp experiments and molecular biology approaches. As we have recently reported, NaCh expression underlying both INaL and INaT remains robust over 5 days in culture in VCMs of the normal dog (18). This time frame is sufficient to silence genes of interest causing a significant NaCh protein level reduction (18). In the present study, we show that HF-related differences in INaL remained during the primary VCMs culture. Indeed INaL density was larger (Fig. 3B) and inactivation kinetics was slower (Fig. 3, C and D) in VCMs from failing compared with normal heart. Therefore, the primary culture of adult dog cardiomyocytes is a suitable model to study molecular mechanisms of INaL regulation in normal and failing VCMs.

Molecular Mechanisms of INaL Modulation by Naβ1 and Naβ2 Subunits

The effects of the β-subunits on Naβ1.5 and other Naβs were extensively studied in the past both in the heterologous expression systems and, recently, in the native heart tissues although with contrasting results (7, 14, 19, 27). Our study was not aimed to solve these controversies but rather to assess whether Naβ1 or Naβ2 modulates INaL in the native NaCh environment of VCMs isolated from both normal and failing hearts.

Fig. 5. Effects of post-transcriptional silencing of Naβ1 and Naβ2 subunits on action potential (AP) of cardiac myocytes from dogs with chronic HF. A: superimposed typical AP traces recorded at stimulation rate 0.25 Hz in cells transfected with control nonsilencing siRNA (control), with Naβ1-siRNA and Naβ2-siRNA, respectively. B: statistical data for AP duration at 90% (APD90) recorded at 3 different stimulation rates of 0.25, 0.5, and 1.0 Hz. Data represent means ± SE pooled from 5 to 15 cells obtained from at least 3 different transfections after 5 days in culture. *Statistical significant difference (P < 0.05, ANOVA followed by Bonferroni’s post hoc test) compared with control.

2) What are manifestations of INaL modulation by SCN1B and SCN2B? A recent study has shown that SCN1B gene silencing caused increased INaL as a result of increased expression of SCN5A in the SCN1B-null mice (14). The biophysical properties of INaL recorded in the SCB1-null mice, however, have not been fully examined, which makes it difficult to compare them with well-known properties of the slowly inactivating INaL in the rat (29) and in humans (our unpublished data). Recently it has been reported that the INaL, produced by Naβ1.1, can be reduced (2) or increased by the Naβ1 (39).

3) How do Naβ1 or Naβ2 subunit interactions with NaCh produce observed INaL changes? Direct interaction between cytoplasmic COOH terminus (CT) domain of Naβ1.1 with Naβ1 and Naβ3 has recently been demonstrated (39) and thus offers possible molecular mechanisms for INaL modulation by Naβ1 found here (if similar interactions exist for Naβ1.5). The role of the CT to regulate Naβ1.5 inactivation has been recently suggested via the Ca2+/calmodulin-dependent interaction with the III-IV linker, responsible for the initial fast inactivation (1, 33), and has been elucidated for INaL.
regulation (20). It has also been suggested that the $\text{Na}_\beta_1$ subunit modulates Na\text{v}1.5 inactivation via interaction with the microtubule cytoskeleton (4).

Although the role of $\text{Na}_\beta_2$ for the function of Na\text{v} is still not clear, the present study is the first to our knowledge to demonstrate $\text{Na}_\beta_2$ effect on $I_{\text{NaL}}$ in the native cardiac cells. Initially, $\text{Na}_\beta_2$ has been implemented in intercellular adhesion and recruitment of a cytoskeleton protein ankyrin to the plasma membrane at sites to cell-to-cell contact (17). Accordingly, a direct role of $\text{Na}_\beta_2$ subunit on the gating of Na\text{v} was not suggested. Na\text{Ch} protein has direct attachments to the submembrane cytoskeleton via ankyrin (1) and can be related to the cytoskeleton-dependent effects on its gating (5, 43). Recently, we reported that heterologously expressed Na\text{v}1.5 did not reveal any effect on $I_{\text{NaL}}$ produced by human Na\text{v}1.5 (19) that can be explained by a lack of coassembly of the Na\text{v}1.5 $\alpha$-subunit with the Na\text{v}2 subunit (49, 50). Accordingly, for the first time here we report evidence of $\text{Na}_\beta_2$ to modulate $I_{\text{NaL}}$ in adult dog VCMs.

The Na\text{Ch} protein consists of the main pore forming $\alpha$-subunit surrounded by the covalently bound Na\text{v}$\beta_1$ and Na\text{v}$\beta_3$ as well as disulfide-linked Na\text{v}$\beta_2$ and Na\text{v}$\beta_4$ subunits (27). The Na\text{v}$\beta_3$ and Na\text{v}$\beta_4$ subunits are highly homologous with their counterparts Na\text{v}$\beta_1$ and Na\text{v}$\beta_2$, respectively, but may have a distinct and opposite function for Na\text{Ch} gating (2, 10, 28, 32, 47). Therefore, besides the aforementioned direct effects on Na\text{Ch}, the deprivation of Na\text{v}$\beta_1$ or Na\text{v}$\beta_2$ subunits from this multiprotein complex may enhance effects of their counterparts. Indeed, heterologous coexpression of Na\text{v}$\beta_4$ with neuronal Na\text{v}1.1 can induce $I_{\text{NaP}}$ (2) or can modulate Na\text{v}1.5-related $I_{\text{NaL}}$ (28). The same idea could be extended for Na\text{v}$\beta_3$ subunit, which may lead to faster inactivation of Na\text{Ch} (10, 32), and contribute to Na\text{v}$\beta_1$ silencing effects on $I_{\text{NaL}}$ in myocytes from HF reported here.

Therefore, elucidation of interactive effects of Na\text{v}$\beta_3$ and Na\text{v}$\beta_4$ on $I_{\text{NaL}}$ in failing heart merits consideration in future studies.

**Physiological Significance**

As mentioned above, $I_{\text{NaL}}$ contribution into HF mechanisms has been demonstrated in experiments where correction of $I_{\text{NaL}}$ in failing cardiomyocytes resulted in 1) rescue of normal repolarization (Figs. 5 and 6), 2) decrease beat-to-beat APD variability, and 3) improvement of Ca$^{2+}$ handling and contractility (22, 25, 41, 42, 44). Accordingly, $I_{\text{NaL}}$ has emerged as a novel target for cardioprotection to treat the failing heart (24, 26, 34). The fact that $I_{\text{NaT}}$ is decreased but $I_{\text{NaL}}$ is increased in HF (21, 23, 25, 42, 45, 48) suggests that not all Na\text{Ch} must be equally targeted. Class I antiarrhythmic drugs that block $I_{\text{NaT}}$ are proarrhythmic in HF because they slow conduction, thus worsening conduction problems (38), and facilitate the development of re-entry. Accordingly, new strategies for treatment must be taken into account considering the aforementioned constraint: the new type of smart drug or modulator should preferentially reduce $I_{\text{NaL}}$, but not $I_{\text{NaT}}$ (40). Therefore, the results of the present study suggest that silencing SCN1B but not SCN2B could be a plausible mechanism to modulate $I_{\text{NaL}}$ in HF with the aim to improve both contractility and rhythm.

**Study Limitations**

Besides being a suitable model for the patch-clamp and molecular biology studies discussed above, the primary culture of adult VCMs has its limitations. A major limitation arises from the notion that over the culturing period VCMs undergo changes in their morphology and protein expression levels including those of ion channels (31) that, in turn, change their...
AP shape and duration. Another possibility to affect AP duration may affect other off-target ion channel expression. We found L-type Ca²⁺ channels remained unaffected (Supplemental Fig. S3). On the other hand, recently it has been shown that siRNA induced silencing of Na,β₁ caused reduction in expression of some potassium channels, which may affect repolarization process, besides I_{NaT} modulation (7). Accordingly, although cell culture well suits the purpose to specifically test modulation of I_{NaT} by Na,β₁ and Na,β₂ subunits (luckily the density of I_{NaT} does not change in control cell cultures), our results in cultured VCMs with regard to AP changes (Fig. 5) should be treated with caution. For example, although the direction of change of AP duration in the cultured cells is the same as expected from the physiological role of I_{NaT} to support the AP plateau given unaltered I_{Cas} (Supplemental Fig. S3), the specific values of the change may not necessarily report those in intact myocardium. Therefore, our respective experimental data with APs in cell culture play only a supportive role in the present study to illustrate that cultured cells maintain electrical excitability due to the robust I_{NaT} and still generate cardiac-like (yet changed) APs. To overcome this limitation and to illustrate physiological relevance of I_{NaT} modulation by Na,β₁ and Na,β₂, we used an E-C coupling model (Fig. 6) to simulate respective AP changes in both normal and failing VCMs. Another limitation of the study is that roles of Na,β₁ and Na,β₂ subunits in I_{NaT} modulation were not evaluated. Elucidation of interactive effects of Na,β₁ and Na,β₂ on I_{NaT} awaits future studies.

Conclusion

Based on our results with a highly effective RNA interference-based method for post-transcriptional silencing SNCAβ1 and/or SNCAβ2 genes in intact ventricular cardiac myocytes, we conclude that Na,β₁ and Na,β₂ exhibit oppositely directed modulation of I_{NaT} in ventricular myocytes of normal and failing dog hearts. We illustrate importance of this modulation to affect AP duration and shape both experimentally (Fig. 5) and in silico (Fig. 6).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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