Chapter 6

Sodium channel remodeling in subcellular microdomains of murine failing cardiomyocytes

Mathilde R. Rivaud, Esperanza Agullo-Pascual, Xianming Lin, Alejandra Leo-Macias, Mingliang Zhang, Eli Rothenberg, Connie R. Bezzina, Mario Delmar*, Carol Ann Remme*

* these authors contributed equally

Journal of the American Heart Association. 2017; 6(12)
Abstract

Background: Cardiac sodium channel (Nav1.5) dysfunction contributes to arrhythmogenesis during pathophysiological conditions. Nav1.5 localizes to distinct subcellular microdomains within the cardiomyocyte, where it associates with region-specific proteins, yielding complexes whose function is location-specific. We here investigated sodium channel remodeling within distinct cardiomyocyte microdomains during heart failure.

Methods and Results: Mice were subjected to 6 weeks of transverse aortic constriction (TAC, n=32) to induce heart failure. Sham-operated mice were used as controls (n=20). TAC led to reduced left ventricular ejection fraction, QRS-prolongation, increased heart mass and upregulation of pro-hypertrophic genes. Whole-cell sodium current (INa) density was decreased by 30% in TAC versus Sham cardiomyocytes. On macropatch analysis, INa in TAC cardiomyocytes was reduced by 50% at the lateral membrane (LM) and by 40% at the intercalated disc (ID). Electron microscopy and Scanning Ion Conductance Microscopy (SICM) revealed remodeling of ID (replacement of (inter-)plicate regions by large foldings), and LM (less identifiable T-tubules and reduced Z-groove ratios). Using SICM, cell-attached recordings in LM subdomains revealed decreased INa and increased late openings specifically at the crest of TAC cardiomyocytes, but not in groove/T-tubules. Failing cardiomyocytes displayed a denser but more stable microtubule network (demonstrated by increased alpha-tubulin and glu-tubulin expression). Super resolution microscopy showed reduced average Nav1.5 cluster size at the LM of TAC cells, in line with reduced INa.

Conclusion: Heart failure induces structural remodeling of the intercalated disc, lateral membrane, and microtubule network in cardiomyocytes. These adaptations are accompanied by alterations in Nav1.5 clustering and sodium current within distinct subcellular microdomains of failing cardiomyocytes.
Clinical Perspective

What is new?

- Heart failure induces ultrastructural changes and sodium current reduction in both the lateral membrane and intercalated disc region of murine cardiomyocytes.
- Failing murine cardiomyocytes display unchanged Na\textsubscript{v}1.5 protein expression, unaltered Na\textsubscript{v}1.5 cluster density but smaller Na\textsubscript{v}1.5 cluster size, accompanied by remodeling of the microtubule network.
- Within the lateral membrane of failing murine cardiomyocytes, reduced sodium current and increased late openings are specifically observed at the crest region, but not in the T-tubules/grooves, indicating microdomain-specific remodeling.

What Are the Clinical Implications?

- Differential remodeling of sodium channels within subcellular microdomains of failing cardiomyocytes may have functional consequences for arrhythmogenesis during heart failure.
- Unraveling the mechanisms underlying microdomain-specific remodeling of sodium channels in failing cardiomyocytes may uncover novel therapeutic strategies aimed at preventing arrhythmias and sudden cardiac death.
Introduction

Cardiac conduction slowing is a major determinant of life-threatening arrhythmias in patients with heart failure.\(^1\)\(^-\)\(^3\) In failing hearts, reduced sodium current (\(I_{\text{Na}}\)) contributes to slowed conduction.\(^4\)\(^-\)\(^6\) \(\text{NaV}_{1.5}\) is the major pore-forming protein of the cardiac sodium channel and is responsible for the fast entry of sodium ions into cardiomyocytes thereby underlying the upstroke of the action potential and mediating cardiac conduction. Recent studies have shown that \(\text{NaV}_{1.5}\) proteins cluster together and localize to distinct “pools” within subcellular microdomains of the cardiomyocyte and associate with region-specific proteins which regulate their function.\(^7\) In the intercalated disc region, \(\text{NaV}_{1.5}\) co-localizes with N-cadherin, ankyrin G, plakophilin-2 and SAP97 while \(\text{NaV}_{1.5}\)-based channels located at the lateral membrane of the cardiomyocyte associate primarily with the dystrophin-syntrophin complex.\(^8\)\(^,\)\(^9\) Remodeling of region-specific proteins may affect \(\text{NaV}_{1.5}\) locally and lead to disturbances in sodium current, conduction abnormalities and arrhythmias.\(^10\)\(^-\)\(^13\)

Classically, sodium channels at the intercalated disc have been considered the most relevant for conduction, but recent evidence indicates a functional role for the lateral membrane sodium channels as well.\(^13\) In this region, functional sodium channels are found in the sarcolemmal crest regions, in the membrane invaginations (grooves) as well as in the T-tubules.\(^14\) The differential function of these distinct pools of channels has not been clearly elucidated. Moreover, it is as yet unknown how these different pools remodel in the setting of heart failure and how they each contribute to the overall reduction in \(I_{\text{Na}}\) during this condition. Using high resolution functional and imaging techniques, we here demonstrate \(\text{NaV}_{1.5}\) cluster (re-)organization and microdomain-specific \(I_{\text{Na}}\) remodeling in failing murine cardiomyocytes.
Methods

Study approval
Animal experiments were carried out in accordance with New York University guidelines for animal use and care and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 58-23, revised 1996).

Induction of heart failure in mice by pressure overload
Male C57/Bl6 mice (Charles River, 10-12 weeks old) were anaesthetized by isoflurane (mean 2.5% in oxygen), intubated with a 20 G polyethylene catheter, ventilated (Minivent Type 845, Harvard apparatus) and placed on a heating pad to maintain body temperature at 37°C. Buprenex (Buprenorphine, 0.05 mg/kg) was injected subcutaneously for postsurgical analgesia. The thoracic cavity was accessed through a small incision at the left upper sternal border in the second intercostal space. A 7-0 silk suture was passed around the aorta between the right innominate and left common carotid arteries. Constriction of the transverse aorta was performed by tying against a 27 G needle, which was subsequently removed. The same procedure was followed in Sham animals, except for the constriction. A pressure gradient (calculated as the ratio between the maximum blood flow velocity of the right carotid over the left carotid artery) of at least 5 was confirmed two days after the procedure using pulsed doppler measurements (Vevo; FujiFilm VisualSonics Inc.).

Echocardiographic and electrocardiographic (ECG) measurements in mice
After 6 weeks of TAC, mice were anaesthetized using isoflurane inhalation (4% for induction, 1–1.5% in 700 mL O2/minute for maintenance) and echocardiographic measurements were recorded as previously described. In a subset of mice (Sham n=5, TAC n=7), this was directly followed by surface ECG recordings. Mice were placed on a heating pad. A standard-lead electrocardiogram (ECG) was recorded with limb electrodes. ECG traces were signal averaged and analyzed for ECG parameters using the LabChart7Pro software (ADInstruments). QRS-duration was calculated from lead III, other parameters from lead I.

Cell dissociation
Adult mouse ventricular myocytes were obtained by enzymatic dissociation following standard procedures. Briefly, after 6 weeks of TAC, mice were injected with 0.1 ml heparin (500 IU/ml intraperitoneally) 20 min before heart excision and anesthetized by carbon dioxide inhalation. Deep anesthesia was confirmed by lack of response to otherwise painful stimuli. Hearts were quickly removed from the chest and placed in a Langendorff column. For cell dissociation, the isolated hearts
were perfused sequentially with low calcium, and an enzyme (collagenase, Worthington) solution. Ventricles were cut into small pieces, and gently minced with a Pasteur pipette. Calcium concentration was increased gradually to normal values. Cardiomyocytes were used on the same day of isolation.

_Patch Clamp Experiments_
Sodium current recordings were conducted either in whole cell configuration or in the cell-attached configuration as described before.\textsuperscript{9,16} Pipette resistance was maintained within the range of 1.9 to 2.1 MΩ, to facilitate reproducibility between experiments. Only recordings obtained under seal resistances higher than 2 GΩ were used in this study.

_Scanning Ion Conductance Microscopy_
Freshly isolated cardiomyocytes were studied by Scanning Ion Conductance Microscopy (SICM), a high resolution (<20nm) technique that allows the 3-dimensional visualization of the surface of live cells. SICM combines noncontact scanning probe microscopy with cell-attached patch clamp, enabling recordings of ion currents at a given location on the lateral membrane of cardiomyocytes. Following surface scanning, Z-groove ratios, indicative of completeness of the Z-grooves, were calculated as followed: Z-groove ratio=length of visible Z-grooves on a scan / total estimated Z-groove length as if they were all complete.\textsuperscript{17} Patch-clamp was performed at a chosen location (i.e crest, groove/T-tubule). Pipet solution contained (in mmol/L): NaCl 148, NaH\textsubscript{2}PO\textsubscript{4} 0.4, MgCl\textsubscript{2} 1, CdCl\textsubscript{2} 0.2, KCl 5.4, HEPES 15, CaCl\textsubscript{2} 1.0, and glucose 5.5, pH 7.4 with NaOH. Cells were maintained in a solution containing the following (in mmol/L): 0.33 NaH\textsubscript{2}PO\textsubscript{4}, 5 HEPES, 1.0 CaCl\textsubscript{2}, and 140 KCl, pH 7.4 with KOH, thus depolarizing the membrane potential to a value estimated to be near zero. To assess the presence of fast inward currents, and to define the unitary current-voltage relation, the membrane under the patch was held at -120 mV and voltage clamp pulses of 1 second were applied every 3 seconds to -30, -40, -50, -60, -70 and -80 mV. The number of channels in a patch (n) was calculated as n=I/(V x g) where g is the single channel conductance. Detailed methods were previously described in Bhargava et al.\textsuperscript{14} Recordings at both locations (i.e crest, groove/T-tubule) were also investigated for the presence of late sodium channel openings, which were defined as stochastic events larger than 1 pA occurring between 200 ms and 1000ms after the pulse.

_Electron Microscopy_
Mice were anesthetized and perfused with 0.1M phosphate buffer containing 2% paraformaldehyde and 2.5% glutaraldehyde and then euthanized by excision of the
heart. The perfused heart was cut into $1\text{mm}^3$ and placed in the same fixative solution at 4°C overnight. After washing with 0.1M phosphate buffer for 30 minutes at room temperature, the tissue was placed in phosphate buffer containing 2% OsO$_4$ / 1.5% potassium ferrocyanide for 1 h at room temperature, washed three times for 5 min in double-distilled H$_2$O (ddH$_2$O) at room temperature and then placed in a filtered solution of 1% thiocarbohydrazide (EMS) in ddH$_2$O for 20 min at room temperature to allow for additional osmium staining. The tissue was then washed three times in ddH$_2$O and then placed in 2% aqueous OsO$_4$ for 30 min at room temperature. Finally, the tissue was washed three times in ddH$_2$O and placed in 1% aqueous uranyl acetate at 4°C overnight. The next day, tissue was washed three times in ddH$_2$O. En bloc lead staining was performed to enhance membrane contrast. A lead aspartate solution was made by dissolving 0.066 g of lead nitrate in 10 ml of 0.003 m aspartic acid. The pH was adjusted to 5.5 with 1N KOH, and the solution was placed in a 60°C oven for 30 min. The lead aspartate solution was filtered, and the tissue was stained at 60°C for 30 min. It was determined that this enhanced osmium staining protocol, combined with en bloc lead staining, was critical for enhancing membrane contrast. The sample was then washed three times in ddH$_2$O, dehydrated in a series of ethanol solutions (30, 50, 70, 85, 95, 100, 100%; 10 min each, on ice) and then placed in ice-cold dry acetone for 10 min, followed by 10 min in acetone at room temperature. The sample was then gradually equilibrated with Durcupan ACM Araldite embedding resin (Electron Microscopy Sciences, EMS, PA) by placing in 25% Durcupan/acetone for 2 h, 50% Durcupan/acetone 2 h, 75% Durcupan/acetone for 2 h, and 100% Durcupan overnight. The tissue was then embedded in fresh 100% Durcupan and placed in a 60°C oven for 48 h to allow Durcupan polymerization and complete the embedding procedure. 60nm thin sections were cut and mounted on 200 mesh copper grids (Electron Microscopy Sciences, Hatfield, PA). Images were acquired using a Philips CM-12 electron microscope (FEI; Eindhoven, The Netherlands) equipped with a Gatan (4k x2.7k) digital camera (Gatan, Inc., Pleasanton, CA).

**Immunocytochemistry**

Freshly isolated cardiomyocytes from Sham or TAC mice were plated on laminin coated coverslips and left to adhere for at least 30 minutes before fixation (4% Formaline). Immunohistochemistry was performed as previously described using N-Cadherin (BD Biosciences, 610921) and Glu-tubulin (Millipore, AB3201) antibodies. Cardiomyocytes were imaged using a Leica SP5 confocal microscope. The glu-tubulin signal was quantified using ImageJ. Three different cytosolic region of interest (ROI) were defined (12µm diameter ROIs): two close to the ID and one in the middle of the cardiomyocyte, always excluding the nuclei. RGB images were split into channels and raw integrated density (sum of pixel values)
was measured in the green channel (glu-tubulin) only in the ROIs defined previously.

**Super-resolution microscopy**

Freshly isolated cardiomyocytes from Sham or TAC mice were plated on laminin coated coverslips and left to adhere for at least 30 minutes before fixation (4% Formaldehyde). Super-resolution microscopy was conducted in a custom-built system, based on simultaneous two-colour direct stochastic optical reconstruction microscopy (STORM). Detailed experimental methods are provided in previous publications. Cluster analysis was automated for standardization following criteria detailed in 19. Lateral membrane cluster analysis was implemented for this study. Lateral membrane clusters were defined as the clusters localized along the lateral edges of the cardiomyocytes.

**Quantitative PCR Assay and analysis**

Total mRNA was isolated from micro-dissected ventricular tissue from Sham or TAC hearts using RNeasy Mini Kit (QIAGEN). 250 μg of mRNA was used as template for reverse transcription using the Reverse Transcriptase-SuperScript II Kit (Invitrogen, Life technologies). Real time RT-PCR was carried out in a Light Cycler apparatus (Roche) using SYBR Green and appropriate mouse primers. The data was analysed with the LinReg program.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hprt-forward</td>
<td>5’_CTTTCCCTGGTTAAGCAGTACAG_3’</td>
<td></td>
</tr>
<tr>
<td>Hprt-reverse</td>
<td>5’_GTCAAGGGCATATCCAACAAAAAC_3’</td>
<td></td>
</tr>
<tr>
<td>Nppa-forward</td>
<td>5’_CCATATTGGAGCAAATCCTGTGT_3’</td>
<td></td>
</tr>
<tr>
<td>Nppa-reverse</td>
<td>5’_TCCTCAGTCTGTCACTCAG_3’</td>
<td></td>
</tr>
<tr>
<td>Acta1-forward</td>
<td>5’_CCATCGGCAATGAGCGTTTC_3’</td>
<td></td>
</tr>
<tr>
<td>Acta1-reverse</td>
<td>5’_ACATGACGTTGTGGCATAACAG_3’</td>
<td></td>
</tr>
<tr>
<td>Scn5a-forward</td>
<td>5’_TTGTCATCCCTCTCCATGGTG_3’</td>
<td></td>
</tr>
<tr>
<td>Scn5a-reverse</td>
<td>5’_AGACGGATGACACGGAAGAG_3’</td>
<td></td>
</tr>
</tbody>
</table>

**Protein isolation and Western Blot**

Sham and TAC ventricles were collected and homogenized in ice-cold RIPA buffer. The tissue lysates were probed by Western blot with Na\textsubscript{v}1.5 (Sigma S0819), alpha-Tubulin (Santa Cruz, sc-5286), EB1 (MAPRE1, Abcam, ab53358), Cx43 (BD Transduction Laboratories, 610061), Calnexin (Calbiochem, 208880), Pan-cadherin (Sigma, C3678) and GAPDH (Fitzgerald Industries International) antibodies. Western blot band intensity was analyzed using Image J software and normalized to loading control.
Statistical analysis
Data are presented as mean ± SEM. Differences between groups were analyzed by unpaired Student’s t-test, nonparametric, parametric or nested ANOVA as appropriate. For the STORM data, a Mann Whitney test was used to test the difference in Na\textsubscript{V}1.5 cluster area. The level of statistical significance was set to p<0.05.
Chapter 6

Results

Cardiac failure phenotype in TAC mice

Of the 32 animals subjected to TAC (with a Doppler ratio above 5), 6 (19%) died suddenly during the course of 6 weeks post-procedure, while none of the Sham mice died (Figure 1A-B). Surviving animals presented signs of heart failure including shortness of breath, a doubling in heart mass and an increase in lung weight as compared to Sham operated animals (Figure 1C). Echocardiographic recordings revealed an enlarged left ventricular chamber in TAC mice with a corresponding increase in left ventricular end-diastolic volume and decrease in left ventricular ejection fraction to 24±1% in TAC as compared to 52±2% in Sham mice (p<0.001) (Figure 1D-E). Surface electrocardiogram recordings revealed a significantly increased heart rate and prolonged PR-, QRS-, and QTc-intervals in TAC versus Sham mice (Table 1). Furthermore, mRNA levels of pro-hypertrophic genes Nppa and Acta1 were upregulated 7- and 4-fold respectively in TAC ventricles as compared to Sham (Figure 1F).

<table>
<thead>
<tr>
<th></th>
<th>Heart rate (BPM)</th>
<th>RR-interval (ms)</th>
<th>PR-interval (ms)</th>
<th>QRS-interval (ms)</th>
<th>QT-interval (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (N=5)</td>
<td>431.3±16.2</td>
<td>139.9±4.9</td>
<td>40.1±0.6</td>
<td>9.8±1</td>
<td>49.4±2.4</td>
</tr>
<tr>
<td>TAC (N=7)</td>
<td>517.0±10.4*</td>
<td>116.3±2.3*</td>
<td>44.7±2.3</td>
<td>13.9±0.8*</td>
<td>61.1±3.1*</td>
</tr>
</tbody>
</table>

Data presented as mean±SEM, *p<0.05 vs. Sham

Decreased sodium current at the lateral membrane and intercalated disc of failing cardiomyocytes

Increased QRS-duration in heart failure is indicative of ventricular conduction slowing. To investigate potential sodium channel remodeling underlying these conduction abnormalities, we first assessed the effect of TAC on whole cell sodium current (I\textsubscript{Na}) characteristics using conventional patch clamp in isolated ventricular cardiomyocytes. Average whole cell I\textsubscript{Na} amplitude was not different between Sham and TAC myocytes (Figure 2A-B and Table 2). However, I\textsubscript{Na} density (current amplitude corrected by cell capacitance) was significantly decreased in failing cardiomyocytes (Figure 2C and Table 2), associated with an increase in cell capacitance in the TAC group (Figure 2D and Table 2). No changes were observed in steady-state voltage dependent activation, inactivation or recovery from inactivation between the groups (Figure 2E-F and Table 2). We next investigated whether the reduced whole-cell I\textsubscript{Na} originated from the mid-section (lateral membrane region; LM) and/or the end of the cell (intercalated disc region; ID).
Figure 1. **Cardiac failure characterization in TAC mice.** (A) Increased Doppler ratio in TAC mice as compared to Sham mice. (B) Kaplan-Meier survival curves of Sham and TAC mice. (C) Cardiac hypertrophy secondary to TAC demonstrated by increased heart (HW) and lung (LW) weights corrected for tibia length (TL) in TAC mice. (D) Typical M-Mode echocardiographic recording in Sham and TAC mice showing decreased contractility, increase walls and chamber sizes in TAC as compared to Sham. (E) Decreased ejection fraction and increased left ventricular end diastolic (LVED) volume in TAC as compared to Sham hearts. (F) Increased expression of pro-hypertrophic genes Nppa and Acta1 corrected for Hprt in ventricular tissue of TAC mice as compared to Sham.
Consistent with previous findings, cell-attached measurements in Sham cardiomyocytes revealed a lower $I_{\text{Na}}$ amplitude and a negative shift in steady-state inactivation in the LM as compared to the ID region (Figure 3A-B, Supplemental Figure 1 and Table 2). In TAC cardiomyocytes, $I_{\text{Na}}$ amplitude was reduced in LM and ID by 50% and 40% respectively (Figure 3A-B and Table 2), without any changes in gating parameters between Sham and TAC. (Figure 3 C-F, and Table 2).

**Microdomain-specific sodium current remodeling within the lateral membrane of failing cardiomyocytes**

Within the LM, two sub-domains can be distinguished, i.e. sarcolemmal crest regions separated by membrane invaginations (grooves) containing T-tubules. We next investigated $I_{\text{Na}}$ alterations in TAC within these LM subdomains using Scanning Ion Conductance Microscope (SICM) combined with cell-attached patch.

**Figure 2. Whole cell current density decreased in TAC cardiomyocytes.** (A) Typical whole cell sodium current ($I_{\text{Na}}$) recordings in Sham and TAC cardiomyocytes. (B) $I$-$V$ relationship of $I_{\text{Na}}$ showing similar current amplitude in Sham and TAC cardiomyocytes. (C) $I$-$V$ relationship of $I_{\text{Na}}$ showing decreased current density (current amplitude corrected for cell capacitance) in TAC cardiomyocytes as compared to Sham cardiomyocytes (*p<0.05 Sham versus TAC, nested ANOVA). (D) Increased cell capacitance in TAC cardiomyocytes. (E) Identical voltage dependence of inactivation and activation of whole cell sodium currents in Sham and TAC. (F) Identical recovery from inactivation in Sham and TAC cardiomyocytes.
Figure 3. Decreased sodium current at the ID and LM of TAC cardiomyocytes. (A) Bright field image of a cardiomyocyte illustrating macropatch recording locations (square at the intercalated disc, ID and triangle at the lateral membrane, LM, scale bar: 10 μm) and typical macropatch $I_{\text{Na}}$ recordings in Sham and TAC cardiomyocytes at the intercalated disc (ID) and lateral membrane (LM). (B) I-V relationship of $I_{\text{Na}}$ showing decreased $I_{\text{Na}}$ in TAC cardiomyocytes as compared to Sham at the LM and ID (*p<0.05 Sham versus TAC, nested ANOVA). (C) Identical voltage dependence of activation and inactivation of ID $I_{\text{Na}}$ in Sham and TAC. (D) Identical recovery from inactivation of ID $I_{\text{Na}}$ in Sham and TAC. (E) Identical voltage dependence of activation and inactivation of LM $I_{\text{Na}}$ in Sham and TAC. (F) Identical recovery from inactivation of LM $I_{\text{Na}}$ in Sham and TAC.
This technique allows to first scan the topology of the cell surface and then to patch with precision the microdomain of interest (crest or groove/T-tubule). As previously reported, LMs of Sham myocytes were organized in a succession of crests and grooves with clearly identifiable T-tubule openings (Figure 4A).\(^2\) Crest-crest distance was approximately \(\sim 2\ \mu\text{m}\) and the Z-groove ratio, indicative of the completeness of the groove, was 85\% (Figure 4C-D). In failing myocytes, the crest-crest distance was unchanged, but the Z-groove ratio was decreased to 63\%, indicating less complete and less deep Z-grooves on the surface of TAC myocytes (Figure 4B-D). Moreover, T-tubule openings were more difficult to identify in failing cardiomyocytes (Figure 4B). Typical examples of cell-attached patch recordings at the crest and groove/T-tubule in Sham and TAC are represented in Figure 5A and B. The initial peak is followed by single-channel events. In both locations we did not observe differences in single-channel conductance between Sham and TAC (Figure 5C-D). The average number of channels per crest recording was 41.2 in Sham while it was only 25.4 in TAC cardiomyocytes. In Sham cardiomyocytes, half of the recordings from the cell crests carried more than 20 open channels (Figure 5E), whereas a lack of open channels (no current) was seen in only a small proportion (~4.5\%) of recordings. In contrast, a larger fraction of crest recordings from TAC myocytes carried no \(I_{\text{Na}}\) (i.e. absence of open channels in ~35\%, \(p=0.012\) vs. Sham), with a lower percentage of recordings containing large currents (comprising more than 50 channels; Sham: 23\% vs TAC: 13\%; Figure 5E). In contrast, and consistent with earlier reports\(^1\), within the groove/T-tubule regions of Sham myocytes, the vast majority of recordings detected no or only small \(I_{\text{Na}}\) (<5 open channels). Groove/T-tubule regions contained on average 3.8 channels per recording in Sham cardiomyocytes and 3.2 channels per recording after TAC. This pattern appeared unchanged in TAC myocytes (Figure 5F). Overall, these results show a remodeling of the lateral membrane region of failing cardiomyocytes, where \(I_{\text{Na}}\) is predominantly reduced in sarcolemmal crest regions.

### Table 2. Whole cell and cell-attached sodium current characteristics from lateral membrane (LM) and intercalated disc (ID) from Sham and TAC cardiomyocytes

<table>
<thead>
<tr>
<th></th>
<th>Capacitance</th>
<th>(I_{\text{Na}}) peak (nA whole, pA attached)</th>
<th>(I_{\text{Na}}) peak (pA/pF)</th>
<th>(V_{1/2}) activation (mV)</th>
<th>(V_{1/2}) inactivation (mV)</th>
<th>Recovery time constant (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (N=4, n=12)</td>
<td>103.9 ± 10.9</td>
<td>-4.1 ± 0.5</td>
<td>-41.7 ± 3.6</td>
<td>-57.7 ± 0.7</td>
<td>-87.9 ± 1.1</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>TAC (N=7, n=14)</td>
<td>157.5 ± 15.9*</td>
<td>-4.3 ± 0.5</td>
<td>-29.6 ± 2.1*</td>
<td>-54.2 ± 0.8</td>
<td>-87.7 ± 0.9</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td><strong>Cell-attached LM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham (N=4, n=12)</td>
<td>-193.0 ± 19</td>
<td>-53.5 ± 1.4</td>
<td>-93.2 ± 0.6†</td>
<td>-10.9 ± 0.5†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAC (N=7, n=14)</td>
<td>-95.3 ± 8*</td>
<td>-52.4 ± 1.1</td>
<td>-93.1 ± 0.7†</td>
<td>11.0 ± 0.6†</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cell-attached ID</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham (N=4, n=12)</td>
<td>-421.3 ± 52.9</td>
<td>-55.7 ± 0.9</td>
<td>-87.4 ± 1.2</td>
<td>6.5 ± 0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAC (N=4, n=7)</td>
<td>-252.2 ± 2.2*</td>
<td>-53.2 ± 1.1</td>
<td>-87.7 ± 1.0</td>
<td>8.2 ± 0.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM, N denotes number of animals, n denotes number of cardiomyocytes

\*p<0.05 vs. Sham  †p<0.005 vs. ID
Sodium channel remodeling in heart failure

Figure 4. Structural remodeling of the lateral membrane of TAC cardiomyocytes. (A) Typical SICM scan and topology profile along the dashed line of a Sham cardiomyocyte lateral membrane (Arrows indicate T-tubule openings). (B) Typical SICM scan and topology profile along the dashed line of a TAC cardiomyocyte lateral membrane. (C) Unchanged crest-crest distance between Sham and TAC. (D) Decreased Z-groove ratio in TAC as compared to Sham.

Increased late sodium channel openings at the crests of failing cardiomyocytes
In addition to peak $I_{Na}$ alterations, enhanced late $I_{Na}$ has also been described in failing cardiomyocytes. As an estimate of late $I_{Na}$ enhancement within specific microdomains, we assessed the numbers of late sodium channel openings (>200ms after the pulse, >1pA stochastic transitions) in the SICM recordings at the LM of Sham and TAC. Figure 6A and B show typical recordings illustrating late sodium channel openings in Sham and TAC at the crest and groove/T-tubule, respectively. We observed an increased number of late openings at the crest of TAC cardiomyocytes as compared to Sham but not in the groove/T-tubule compartment (Figure 6C-D), indicating location-specific late openings.

Structural modifications of the intercalated disc in failing cardiomyocytes
Proper insertion and function of $\text{Na} v 1.5$ requires an intact membrane structure and organization. Hence, during HF, membrane remodeling may contribute to $I_{Na}$ reduction within distinct subcellular microdomains. Our SICM measurements indeed demonstrated membrane remodeling at the LM, including reduced depth of the grooves. We next investigated structural alterations of the ID membrane in ventricular tissue using Electron Microscopy (EM). IDs in Sham tissue featured clear plicate (P) and inter-plicate (IP) regions while the IDs of TAC cardiomyocytes consisted of a succession of large folds (Figure 7A-C). The last sarcomere of failing cardiomyocytes flanking the ID was disordered with visible extended filaments (circle; bottom panel in Figure 7B). Vacuoles were clearly identifiable next to the ID membrane in TAC (arrows, Figure 7C). Thus, significant (ultra)structural changes were evident in ID of failing cardiomyocytes.
Figure 5. Decreased sodium current at the crest of TAC cardiomyocytes. (A) Typical sodium current recordings at the crest of Sham and TAC lateral membrane. Brackets highlight single channel events. (B) Typical sodium current recordings in the groove/T-tubule of Sham and TAC lateral membrane. Brackets highlight single channel events. Please mind the scale. (C) Identical unitary channel conductance in crest in Sham and TAC. Number of events at Vm= -50, -60, -70, -80 are for Sham 68, 138, 139 and 85 and for TAC 29, 58, 87, 114. (D) Identical unitary channel conductance in groove/T-tubule in Sham and TAC. Number of events at Vm= -50, -60, -70, -80 are for Sham 73, 76, 62 and 72 and for TAC 66, 67, 58 and 66. (E) Decreased numbers of channels at the crest of TAC cardiomyocytes when compared to TAC (Chi-square test p=0.012 for “0”). (F) Identical number of open channels in groove/T-tubule in Sham and TAC cardiomyocytes.
Sodium channel remodeling in heart failure

Figure 6. Increased late opening of sodium channels at the crest of TAC cardiomyocytes. (A) Typical recordings showing late sodium channel openings in Sham and TAC crests. Magnification of the rectangular red boxes are shown below. (B) Typical recordings showing late sodium channel openings in Sham and TAC groove/T-tubule. Magnification of the rectangular red boxes are shown below. (C) Increased number of late openings in TAC crest as compared to Sham crest. (D) Identical number of late openings in Sham and TAC groove/T-tubule.

Microtubule network alterations in failing cardiomyocytes
A number of mechanisms potentially underlie the observed $I_{Na}$ remodeling in the various subcellular microdomains of failing myocytes. $NaV_{1.5}$ is trafficked to and delivered into the cell membrane by the microtubule (MT) network. Specific sites of delivery are determined by macro-molecular complexes comprised of anchoring and microtubule capture proteins. In TAC hearts, increased protein expression levels of the MT protein alpha-tubulin and plus-end microtubule capture protein EB1 were observed, indicating a denser microtubule network and a potential increase in MT capture sites at the cell membrane (Figure 8A-B). Immunoreactive signal intensity for detyrosinated alpha-tubulin (glu-tubulin) was also significantly increased after TAC (Figure 8C-D), confirming increased MT density but also indicating a less dynamic MT network. This denser yet remodeled MT network suggests altered trafficking and/or delivery of $NaV_{1.5}$ to the cell membrane of failing cardiomyocytes.

Reorganization of $NaV_{1.5}$ clusters in failing cardiomyocytes
We next sought to define the impact of heart failure on $NaV_{1.5}$ distribution and clustering at the membrane. To this end we utilized super-resolution localization microscopy (STORM) to resolve the molecular organization of $NaV_{1.5}$ in fixed
Figure 7. **ID structural and molecular alterations in TAC cardiomyocytes.** (A) Electron microscopy image of an intercalated disc from a Sham cardiomyocyte featuring clear plicate (P) and inter-plicate (IP) structures (Scale bar: 2μm). (B) Electron microscopy image of a TAC intercalated disc showing large disorganized deflections and extended filaments in the last sarcomere (dashed oval) (Scale bar: 2μm). (C) Electron microscopy image of an entire TAC intercalated disc with detail (black rectangle) showing large disorganized deflections and identifiable vacuoles (arrows) (Scale bar left image: 5μm, scale bar right image: 2.5μm).

We note that no differences in *Scn5a* mRNA levels or total Na\(_V\)1.5 protein levels were observed between Sham and TAC ventricular tissue (Figure 9A-C). Figure 9D shows examples of immune-reactive signals of Na\(_V\)1.5 and N-Cadherin (marking the intercalated disc) detected by STORM in Sham and TAC cardiomyocytes. The N-Cadherin signal on STORM images of TAC cardiomyocytes demonstrated a loss of plicate and inter-plicate structures as compared to Sham myocytes, in accordance with the EM images of the ID in Figure 7. Given this disorganized pattern, it was not possible to accurately delineate the cell end, thus precluding reliable calculations for Na\(_V\)1.5 cluster size and density at the ID of TAC myocytes. To measure the area and the density of Na\(_V\)1.5 clusters at the LM, we traced a 1 μm thick line delineating the lateral membrane, perpendicular to the N-Cadherin signal. The line was traced along the green clusters at the cell edge. In total, 289 clusters from 11 images of Sham cardiomyocytes and 272 clusters from 9 images of TAC cardiomyocytes were...
incorporated in the analysis. The minimum area defined as a cluster was 2400 nm$^2$ and cluster sizes ranged up to 404400 nm$^2$ in Sham and 230000 nm$^2$ in TAC myocytes. As shown in Figure 9E, $\text{Na}_V1.5$ cluster density at the LM was not significantly different between Sham and TAC. However, $\text{Na}_V1.5$ clusters at the lateral membrane were significantly smaller in TAC as compared to Sham, as indicated by a shift towards smaller clusters in TAC cardiomyocytes (Mann-Whitney test, $p=0.0023$, Figure 9F,G). Hence, $\text{Na}_V1.5$ cluster density at the LM was maintained in failing cardiomyocytes, but at the expense of cluster size.

Figure 8. Microtubule remodeling in TAC cardiomyocytes. (A-B) Increased alpha-tubulin and EB1 protein expression corrected for Calnexin (loading control) in TAC as compared to Sham ventricular tissue on Western blot analysis. (C-D) Increased glu-tubulin expression in TAC as compared to Sham cardiomyocytes on immunohistochemistry analysis (Scale bar: 20μm).
Figure 9. Sodium channel remodeling in TAC cardiomyocytes. (A) Similar Scn5a mRNA expression corrected for Hprt in Sham and TAC ventricular tissue on qPCR analysis. (B-C) Similar Na\textsubscript{V}1.5 protein expression corrected for Gapdh (loading control) in Sham and TAC ventricular tissue on Western blot analysis. (D) Typical example of STORM images labeled for Na\textsubscript{V}1.5 (green) and N-Cadherin (purple) in Sham and TAC cardiomyocytes (Scale bar: 5\textmu m). (E) Similar number of Na\textsubscript{V}1.5 cluster x area at the lateral membrane (LM) in Sham and TAC cardiomyocytes and decreased Na\textsubscript{V}1.5 cluster size at the LM of TAC as compared to Sham cardiomyocytes. (F-G) Distribution of Na\textsubscript{V}1.5 cluster sizes in Sham and TAC.
Discussion

We here investigated for the first time sodium current remodeling and sodium channel cluster (re-)organization within different subcellular microdomains of failing cardiomyocytes using nanometer precision techniques. We demonstrate a similar reduction in $I_{Na}$ at the lateral membrane (LM) and intercalated disc (ID) in the setting of (ultra)structural membrane alterations and remodeling of the microtubular network. Our data furthermore provides insight into subdomain-specific $I_{Na}$ reduction and NaV1.5 cluster remodeling at the LM of failing cardiomyocytes.

Remodeling of sodium current in heart failure

Sodium current reduction is well established in heart failure,4-6 but the possibility that sodium channel density and/or function is differentially remodeled within the various subcellular compartments (microdomains) of failing cardiomyocytes has not been previously addressed. Sodium channel clusters in various cardiomyocyte microdomains exhibit distinct current amplitudes and gating properties.9 Studies in animal models have shown differential contribution of distinct subcellular sodium channel pools to global conduction in the heart.10-12 Channels at the ID are considered the most functionally relevant for conduction, and specific reduction of ID sodium current in PKP2 and CAR deficient models have been shown to modulate myocardial electrical properties and arrhythmogenesis.11,12,16 However, loss of channels specifically at the lateral membrane affects conduction anisotropy through preferential conduction slowing in the transverse as opposed to longitudinal direction.13 While these observations indicate a differential functional role for channels within specific microdomains, little is known about how channels in various subdomains are remodeled during pathophysiological conditions. Our current results demonstrate a similar decrease in $I_{Na}$ within the ID and LM of failing murine cardiomyocytes. This global $I_{Na}$ reduction is in line with previously reported ventricular conduction slowing in both the longitudinal and transversal directions in failing hearts26, and together with structural remodeling occurring within the failing myocardium, may set the stage for arrhythmogenesis. Remodeling of other ion channels likely also contributes to arrhythmogenesis during heart failure. While microdomain-specific remodeling of L-type Ca$^{2+}$ channels has been described in failing cardiomyocytes,27 similar research on potassium channel remodeling remains to be performed.

Sodium channel remodeling at the LM of failing cardiomyocytes

Using super-resolution microscopy we observed unchanged NaV1.5 cluster density yet reduced cluster size at the LM of failing cardiomyocytes. Since smaller NaV1.5 clusters have been linked to reduced functionality, the observed decreased cluster
size at the LM of failing myocytes readily explains the reduction in $I_{\text{Na}}$ measured within this region.\textsuperscript{28} The reduction in sodium current amplitude recorded at the LM using the macropatch method was larger than the observed reduction in whole cell current density. This suggests a non-linear relation between the increase in membrane surface (cell size) and the cluster re-organization at the LM of failing cardiomyocytes. STORM analysis did not allow us to distinguish clusters at the crest from those at the groove/T-tubule. Nevertheless, SICM recordings at the LM indicated that $I_{\text{Na}}$ is primarily decreased at the crests of failing cardiomyocytes, and unaffected in the groove/T-tubule microdomain, indicating microdomain-specific remodeling secondary to TAC. In addition, there were significantly more recordings carrying no current in TAC, again suggesting the presence of non-functional clusters at the LM. In line with other studies demonstrating T-tubule remodeling in heart failure, we observed less deep Z-grooves in TAC cardiomyocytes in addition to less clearly identifiable T-tubule openings. Apart from the cardiac sodium channel Na\textsubscript{V}1.5, neuronal sodium channel isoforms (i.e., Na\textsubscript{V}1.1, Na\textsubscript{V}1.3, and Na\textsubscript{V}1.6) are also thought to populate the T-tubules.\textsuperscript{29-32} Studies concerning expression and function of neuronal sodium channel isoforms during HF have shown varied results, most likely due to variations in species (rat, rabbit, dog) and HF model (pressure and/or volume overload, embolizations) used, with the most consistent observation comprising an upregulation of Na\textsubscript{V}1.1.\textsuperscript{5,23,33} However, we observed unaltered $I_{\text{Na}}$ gating properties at the LM of failing myocytes in addition to unchanged single-channel conductance in both the crest and groove/T-tubule microdomains, arguing against a change in neuronal sodium channel function. Thus, the observed reduction in $I_{\text{Na}}$ at the LM of failing cardiomyocytes is likely predominantly caused by a decrease in Na\textsubscript{V}1.5-based channels, in particular those located at the crest. Increased late sodium current is another key feature of heart failure and contributes to arrhythmogenesis.\textsuperscript{23} Interestingly, we observed location-specific increased late openings of sodium channels at the crest of failing cardiomyocytes but not in the groove/T-tubule region. Whether this increase represents an arrhythmogenic substrate in the particular conditions of our experiments, remains to be determined.

Potential role for microtubule network remodeling

Similar to natural growth occurring during cardiomyocyte development, growth during cardiac remodeling is achieved by the insertion of sarcomeres flanking the ID.\textsuperscript{34} Our data show an unchanged crest-crest distance of $\sim$2 $\mu$m in TAC cardiomyocytes corresponding to the sarcomere length of quiescent myocytes. Since cell size was increased (increased cell capacitance, Figure 3C), sarcomeres were likely inserted during the development of hypertrophy in TAC hearts, as described previously by Wilson and coworkers in dilated cardiomyopathy.\textsuperscript{34}
Sodium channel remodeling in heart failure

Whereas number of sarcomeres and cell crests per cell increased, the mRNA and protein expression and density of sodium channel clusters remained unchanged. This indicates that the available Na\textsubscript{V}1.5 channels have been redistributed along the LM at existing and newly formed crest anchoring points, albeit with less channels per cluster (as indicated by reduced cluster size, and less channels within recordings); some of these smaller clusters may even not be functional at all, since we observed a significant increased number of recordings in TAC myocytes carrying no current. Smaller cluster size yet unaltered cluster density in TAC as compared to Sham suggests a re-direction and re-distribution of channels to the newly formed sarcomeres at the lateral membrane in TAC cardiomyocytes. A number of studies have previously shown that Nav1.5 channels are delivered to their final destination on the membrane through the microtubule network.\textsuperscript{19,35} Although microtubule network density was increased in TAC cardiomyocytes, the observed increased de-tyrosinated tubulin (i.e. glu-tubulin) levels indicate that the microtubule network is less dynamic,\textsuperscript{25} and thus potentially less efficient.\textsuperscript{36-38} It is therefore reasonable to speculate that the increased glu-tubulin observed in our experiments is associated with impaired Nav1.5 delivery at any sub-cellular location. Moreover, microtubule densification may also have contributed to the observed T-tubular remodeling in failing cardiomyocytes.\textsuperscript{39}

Remodeling of the intercalated disc in failing cardiomyocytes

In failing cardiomyocytes the ID presented overt (ultra)structural remodeling. Electron microscopy images revealed extended membrane folds at the ID of TAC myocytes, in addition to disorganization of the plicate and inter-plate regions. These increased interdigitations of the ID membrane likely contributed to an enlarged 3-dimensional ID surface in failing myocytes\textsuperscript{34,40,41} and an increased spatial complexity of the overall ID area, with membrane foldings tightly packed next to each other. In this complex space, the separation between membrane foldings can be less than the distance that can be resolved using super-resolution. For example, two clusters on two different ID membrane locations could appear as one if located within 40nm of each other, hence impeding a reliable measurement of Na\textsubscript{V}1.5 cluster size and density quantification. Nevertheless, our observation that I\textsubscript{Na} is reduced at the ID is in line with Na\textsubscript{V}1.5 cluster remodeling at the ID secondary to (ultra)structural changes and microtubule network alterations, similar to the LM.

EB proteins are known to modulate dynamic transition of the microtubules plus-end (growth and shrinkage)\textsuperscript{42} The observed higher expression of the microtubule plus end tracking protein EB1 may also constitute a compensatory mechanism to render the plus end of microtubule more dynamic to maintain delivery Na\textsubscript{V}1.5 to the membrane. Despite this, some sodium channels may
actually not have reached the ID membrane and may not have functionally contributed to sodium current. Moreover, successful anchoring in the membrane does not necessarily guarantee normal functioning channels, and alterations in NaV1.5 partner proteins, such as Cx43, may further compromise channel functionality.

Potential additional mechanisms modulating sodium current in failing cardiomyocytes
In addition to membrane and microtubule network remodeling, other events during heart failure such as altered splicing, glycosylation or changes in neuronal sodium channel composition may also contribute to $I_{Na}$ reduction at the ID and/or LM. Splicing events in heart failure generate multiple sodium channel protein isoforms, some of which lead to less functional channels and modify the gating properties of the overall sodium current.\[^{33,44}\] Glycosylation of the sodium channel is known to modulate $I_{Na}$ and its gating properties, and underlies arrhythmogenesis in heart failure.\[^{45,46}\] While such sodium channel alterations could have potentially contributed to the TAC-induced $I_{Na}$ reduction in the various microdomains, the unaltered $I_{Na}$ gating properties observed after TAC makes this unlikely.

Considerations and limitations
While the super-resolution microscopy employed in this study allowed for localization of fluorescently-labelled proteins with a resolution of several nanometers, our ability to assign position and dimensions to clusters in relation to the cell membrane remain limited, particularly when applied as two-dimensional optical sections of complex three-dimensional objects. Moreover, our findings are limited to the case of pressure overload in mice, and are not necessarily generalizable to all cases of heart failure. Heart failure itself may arise from a large variety of causes, and differences in remodeling mechanisms exist between pressure or volume overload, myocardial infarction or pacing which may each contribute differently to sodium channel (dys)function. These mechanisms likely also differ depending on the stage of the disease.\[^{47-50}\] Hence, while our current observations on $I_{Na}$ remodeling are not necessarily applicable to other models or stages of heart failure, they highlight a potential arrhythmogenic change in the electrophysiological profile of a heart subjected to pressure overload, a common etiology of heart failure.

Conclusion
Heart failure induces structural remodeling of the intercalated disc, lateral membrane, and microtubule network in ventricular cardiomyocytes. These alterations are accompanied by alterations in NaV1.5 clustering and sodium
currents within distinct subcellular microdomains of the failing cardiomyocyte. Sodium current is equally reduced at the intercalated disc and lateral membrane regions of failing murine cardiomyocytes. In the lateral membrane, sodium current reduction is microdomain-specific with decrease primarily at the crests, associated with a reduction in Na\textsubscript{V}1.5 cluster size.

Acknowledgements

The authors are grateful to A. Shekhar for sharing his expert knowledge on echocardiographic measurements and to L. Beekman for designing the primers for qPCR experiments.

Sources of Funding

This work was funded by an Innovational Research Incentives Scheme Vidi grant from ZonMw (grant no. 91714371, to C.A.R.), by the Dutch Heart Foundation/CVON (project PREDICT, CVON2012-10 to C.R.B.), the Dutch Heart Foundation (NHS2010/B201 to C.A.R.), the American Heart Association (AHA postdoctoral fellowship 15POST25550087 to E.A.P) and by the National Institutes of Health (RO1-GM57691, RO1-HL134328 and RO1-HL136179 to M.D).
References


142


34. Wilson AJ, Schoenauer R, Ehler E, Agarkova I, Bennett PM. Cardiomyocyte growth

143


