Cardiac Sodium Channel Na\textsubscript{\text{1.5}} Is Regulated by a Multiprotein Complex Composed of Syntrophins and Dystrophin

Bruno Gavillet, Jean-Sébastien Rougier, Andrea A. Domenighetti, Romina Behar, Christophe Boixel, Patrick Ruchat, Hans-Anton Lehr, Thierry Pedrazzini, Hugues Abriel

Abstract—The cardiac sodium channel Na\textsubscript{\text{1.5}} plays a key role in cardiac excitability and conduction. The purpose of this study was to elucidate the role of the PDZ domain-binding motif formed by the last three residues (Ser-Ile-Val) of the Na\textsubscript{\text{1.5}} C-terminus. Pull-down experiments were performed using Na\textsubscript{\text{1.5}} C-terminus fusion proteins and human or mouse heart protein extracts, combined with mass spectrometry analysis. These experiments revealed that the C-terminus associates with dystrophin, and that this interaction was mediated by alpha- and beta-syntrophin proteins. Truncation of the PDZ domain-binding motif abolished the interaction. We used dystrophin-deficient mdx\textsuperscript{5cv} mice to study the role of this protein complex in Na\textsubscript{\text{1.5}} function. Western blot experiments revealed a 50% decrease in the Na\textsubscript{\text{1.5}} protein levels in mdx\textsuperscript{5cv} hearts, whereas Na\textsubscript{\text{1.5}} mRNA levels were unchanged. Patch-clamp experiments showed a 29% decrease of sodium current in isolated mdx\textsuperscript{5cv} cardiomyocytes. Finally, ECG measurements of the mdx\textsuperscript{5cv} mice exhibited a 19% reduction in the P wave amplitude, and an 18% increase of the QRS complex duration, compared with controls. These results indicate that the dystrophin protein complex is required for the proper expression and function of Na\textsubscript{\text{1.5}}. In the absence of dystrophin, decreased sodium current may explain the alterations in cardiac conduction observed in patients with dystrophinopathies. (Circ Res. 2006;99:407-414.)

Key Words: Duchenne dystrophy • dystrophin • ECG • mouse • sodium channels • syntrophin

The main cardiac voltage-gated sodium channel, Na\textsubscript{\text{1.5}}, generates the fast depolarization of the cardiac action potential, and plays a key role in cardiac conduction. Its importance for normal cardiac function has been exemplified by the description of numerous naturally occurring genetic variants of the gene SCN5A, which encodes Na\textsubscript{\text{1.5}}, that are linked to various cardiac diseases.\textsuperscript{1} Among them, the congenital long QT syndrome type-3 and the Brugada syndrome are caused by gain or loss-of-function of Na\textsubscript{\text{1.5}}, respectively.\textsuperscript{1} Na\textsubscript{\text{1.5}} is the pore-forming α-subunit protein of the cardiac sodium channel. It has a molecular weight of \textasciitilde220 kDa, and may be associated with at least 4 types of auxiliary small (30 to 35 kDa) β-subunits. Recently, several proteins that bind directly to Na\textsubscript{\text{1.5}} have been described.\textsuperscript{2} However, in most cases the physiological relevance of these interactions remains poorly understood, mainly because of a lack of appropriate animal models. With the exception of ankyrin-G,\textsuperscript{3} all these partner proteins interact with the 243-residues-long intracellular C-terminal domain of the channel which contains several protein-protein interaction motifs.\textsuperscript{2} Among them, the last three residues of Na\textsubscript{\text{1.5}} (2014-Ser-Ile-Val-2016) constitute a PDZ domain-binding motif to which syntrophins and dystrophin have been shown to interact directly or indirectly, respectively.\textsuperscript{4–6} However, thus far, the role of these interacting proteins in the heart has never been investigated.

In this study, by performing mass spectrometry-based protein identification using human and rodent cardiac lysates, we could confirm that dystrophin and syntrophin proteins are interacting specifically with the PDZ domain-binding motif of Na\textsubscript{\text{1.5}}. Biochemical and electrophysiological studies using cardiac tissue and cells of dystrophin-deficient mdx\textsuperscript{5cv} mice (an animal model of Duchenne muscular dystrophy [DMD]) revealed that Na\textsubscript{\text{1.5}} protein content as well as Na\textsubscript{\text{1.5}}-mediated current (\(I_{\text{Na}}\)) were decreased in the absence of dystrophin. Finally, ECGs of mdx\textsuperscript{5cv} mice showed alterations characteristic of conduction defects. These findings suggest that Na\textsubscript{\text{1.5}} is part of a multiprotein complex in which dystrophin and syntrophin proteins play an important role in its expression level. Moreover, these results provide the pathophysiological basis for some of the ECG abnormalities seen in DMD patients, and other dystrophinopathy patients, in whom cardiac arrhythmias and conduction defects may lead to sudden death.\textsuperscript{7}

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Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Animals

Wild-type C57BL/6 mice, used as control, were purchased at Janvier (Le Genest St Isle, France), and C57BL/6Ros-5Cv (mdx<sup>5Cv</sup>) mice (Jackson laboratories, Bar Harbor, Me) were raised in our department. Male mice at an age of 10 to 14 weeks were used in this study. All animal procedures were performed in accordance with the Swiss laws.

Cardiac Tissue Samples

The experimental procedures were approved by the ethical commission on clinical research of the faculty of medicine of the University of Lausanne. Human right atrial sample was collected from patients in normal sinus rhythm undergoing coronary bypass surgery. Mice were anesthetized and heart ventricles were excised, rinsed with ice cold PBS and frozen in liquid nitrogen. Fresh human atrial appendage or frozen mouse ventricle was transferred into lysis buffer. Tissues were then homogenized using a Polytron and a Teflon homogenizer. Triton X-100 was added to a final concentration of 1% and solubilization occurred by rotating for 1 hour at 4°C. The soluble fraction from a subsequent 15-minute centrifugation at 13 000g (4°C) was used for the experiments.

Mass Spectrometry Peptide Analysis

SDS-PAGE gels were stained with SYPRO dye (Molecular Probes, Eugene, Ore). The gel was visualized on a UV-light transilluminator. The bands of interest were excised and sent to the Protein Analysis Facility of Lausanne where they were submitted to trypsin digestion, liquid chromatography and tandem mass spectrometry.

Pull-Down Assays

The cDNAs encoding the 66 last amino acids of Na<sub>v</sub>1.5 WT, Kir2.1 and cystic fibrosis transmembrane conductance regulator (CFTR) were expressed in E. coli BL21 bacteria. The cDNAs were cloned into pGEX-4T1 plasmid and the GST fusion proteins were purified using *Escherichia coli* GST-pull-down assay. GST-pull-down assay of solubilized fractions of ventricular lysate were performed using GST-sepharose beads containing either GST or the GST fusion proteins. After 2-hour rotation and washing, bound proteins were detected by Western blot.

Western Blot and Antibodies

Western blotting conditions and the specificity of the polyclonal Na<sub>v</sub>1.5 antibody (ASC-005; Alomone) has been previously described. A control Western blot using the peptide antigen showing the specificity of the signal is presented in the online data supplement. The polyclonal antibody K<sub>2.1</sub> (APC-026) was purchased from Alomone (Jerusalem, Israel). Polyclonal connexin-43 antibody (71-0700) was obtained from Zymed (San Francisco, Calif). Monoclonal pan-syntrophin antibody (MA1–745) was from Biovendor (Villmerken, Austria). Monoclonal dystrophin antibody (MANDYS8) was from SIGMA (Buchs, Switzerland). Monoclonal pan-syntrophin antibody (MA1–745) was purchased from Affinity Bioreagents (Golden, USA). Polyclonal α<sub>1</sub>-<sub>β</sub>-<sub>γ</sub>-syntrophin antibodies were kindly provided by M. Schaub (University of Zurich), and γ<sub>1</sub>- and β<sub>2</sub>-syntrophin antibodies were from Santa Cruz Biotechnology (Santa Cruz, Calif). Polyclonal Ca<sub>1.2</sub> antibody was a gift from J. Hell (University of Iowa), Na,K-ATPase antibody was a gift from K. Geering (University of Zurich) and Nedd4–2 antibody was a gift from O. Staub (University of Lausanne).

TaqMan Real-Time Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from whole ventricles according to described protocol in. cDNA was synthesized from 1 μg of total RNA using the M-MLV reverse transcriptase (Q-Biogene EMMLV100; Irvine, Calif). Fifty nanograms of cDNA combined with 1× TaqMan Universal Master Mix (Applied Biosystems, Foster) and 1 μL of either Na<sub>v</sub>1.5, K<sub>2.1</sub> or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (Applied Biosystems, respectively, Mm00451971, Mm00434616 and Mm99999915) were loaded into each well. GAPDH was used as reference gene for normalizing the data.

Isolation of Cardiac Myocytes and Patch-Clamp Experiments

Adult mouse ventricular myocytes were isolated as described. Whole-cell configuration of the patch-clamp technique was used to record IC<sub>0</sub>. Experiments were performed at room temperature (22°C to 23°C), using a VE-2 (Aelamic Instruments) amplifier allowing the recording of large sodium currents. Pipettes (tip resistance 1 to 2 MΩ) were filled with a solution containing 60 mmol/L aspartic acid, 70 mmol/L cesium asparte, 1 mmol/L CaCl<sub>2</sub>, 1 mmol/L MgCl<sub>2</sub>, 10 mmol/L HEPES, 11 mmol/L EGTA, and 5 mmol/L Na<sub>a</sub>ATP (pH was adjusted to 7.2 with CsOH). Myocytes were bathed with a solution containing 10 mmol/L NaCl, 120 mmol/L NMDG-Cl, 2 mmol/L CaCl<sub>2</sub>, 1.2 mmol/L MgCl<sub>2</sub>, 5 mmol/L CsCl, 10 mmol/L HEPES, and 5 mmol/L Glucose (pH was adjusted to 7.4 with CsOH).

ECG

The mouse ECG was recorded as previously described. Corrected QT interval (QT<sub>c</sub>) was calculated according to Mitchell et al. The mouse ECG was recorded using the magic wand tool and the select similar command, all dark-stained nuclei were selected and their surface quantified by the histogram tool (given in pixel per inch, and then translated into mm<sup>2</sup> according to the total magnification of the image). The mean nuclei size was calculated by the division of the total nuclear area by the number of nuclei at counted using a commercially available plug-in (count marks). The image processing toolkit; Reindeer Games, Charlotte, NC) and given in mm<sup>2</sup>. In the absence of notable tissue fibrosis, edema, or prominent intramycocardial vessels, the cytoplasmic area was calculated as the total area of the 20× field minus the nuclear area (see above) then divided by the number of nuclei assessed as described.

Statistical Analyses

Data are represented as mean values±SEM. Two-tailed Student t test was used to compare means. Statistical significance was set at P<0.05.

Results

C-Terminal Domain of Na<sub>1.5</sub> Interacts With Dystrophin and Syntrophin Proteins

Recently, several proteins have been described to interact with specific protein–protein interaction motifs of the C-terminal domain of Na<sub>1.5</sub>. Using a mass spectrometry-based approach with GST-fusion proteins of the last 66 residues of Na<sub>1.5</sub> C-terminus (Figure 1A), we searched for proteins extracted from human right atrial appendage samples interacting specifically with the PDZ domain-binding consensus motif formed by the last three Ser-Ile-Val (SIV) amino acids. The SDS-page presented in Figure 1B shows a high-molecular-weight protein that was reproducibly pulled down using the WT fusion protein, but not with a protein lacking the SIV residues (ΔSIV). This protein was identified as dystrophin in human atrial samples (Table 1) as well as in...
mouse and guinea pig ventricular samples, with a high degree of confidence. Because dystrophin is known to frequently interact with partner proteins via syntrophin adapter proteins,12 we performed an additional experiment in which we examined by mass spectrometry the last 66 amino acids of Na,1.5 with the PY-motif (PPXY) and PDZ domain-binding motif (SIV). ΔSIV corresponds to the last 66 amino acids carrying the S2014stop mutation, and YA, to the last 66 amino acids with the Y1977A mutation. Pull-down experiment realized by incubating the soluble fractions of a human atrial appendage lysate with GSH-Sepharose beads containing either the GST protein alone (GST) or with two Na,1.5 GST fusion proteins: WT and ΔSIV. Pulled-down fractions and 80 μg of appendage lysate (input) were loaded on a SDS-page gel, which was stained using the Sypro dye. The arrow indicates a band at high molecular weight only present in the fraction pulled-down with the WT fusion protein. This band was excised, analyzed by mass spectrometry and identified as being dystrophin (Table 1). C. Western blots of pulled-down fractions performed on mouse ventricular lysate of either control or mdx5cv mice as indicated. Mouse ventricular lysates were mixed with GSH-Sepharose beads containing either the GST protein alone (GST) or with three Na,1.5 GST fusion proteins: WT, YA, or ΔSIV. Eighty μg of control and mdx5cv ventricular lysate were loaded in the input lane. ΔSIV fusion protein is able to bind Nedd4-2, which is a protein known to interact with the Na,1.5 PY-motif. The bottom panel is a Ponceau staining of a representative nitrocellulose membrane indicating that similar amount of GST and GST fusion proteins were used for the different pull-downs.

In this set of experiments, we also performed pull-down experiments using a fusion protein in which the Na,1.5 PY-motif was mutated (Y1977A; Figure 1A) because this motif is known to interact with WW-domains similar to the one present in dystrophin.13 However, as depicted in Figure 1C, dystrophin also interacted with the PY-motif mutated protein, indicating that this motif is not likely to play a key role in the interaction of dystrophin with Na,1.5. Moreover, it is also apparent that neither dystrophin nor syntrophin proteins bind to the SIV-truncated protein (Figure 1C). The endogenous mouse ubiquitin-protein ligase Nedd4-2, previously shown to bind to the PY-motif of Na,1.513 was pulled-down with the WT and the SIV-truncated fusion proteins, which attests for the integrity of the SIV-truncated protein (Figure 1C). Using isomrf-specific antibodies recognizing α1-, β1-, and β2-syntrophin proteins, we found that all three were pulled-down with the WT Na,1.5 C terminus fusion protein. We also tested two antibodies recognizing γ1- and γ2-syntrophin, but these two proteins could not be detected in the mouse cardiac lysates (not shown).

**Na,1.5 Protein Is Specifically Decreased in mdx5cv Cardiac Tissue**

We then performed Western blot experiments using ventricular lysates of mdx5cv and control mice to study the consequences of the dystrophin deficiency on Na,1.5 protein level. Figure 2A illustrates that when dystrophin was absent, a consistent reduction of the Na,1.5 protein level was detected in four mdx5cv mouse hearts. This phenomenon was specific for Na,1.5 because we did not observe any other obvious decrease in protein levels of major membrane proteins such as the L-type Ca2+ channel (Ca,1.2), connexin-43, and α-subunit of the Na-K,ATPase. The protein levels of several heart extracts were quantified by digital densitometry, and Figure 2B shows that the Na,1.5 content was decreased by 50±9% in mdx5cv hearts compared with controls. With the exception of a small, albeit statistically significant, 14±4% decrease in the level of K,2.1 in mdx5cv hearts, the expression of all other tested proteins was not altered in the absence of dystrophin. It could have been hypothesized that this decrease in Na,1.5 amount in mdx5cv hearts may have been caused by a reduction of the SCN5A mRNA level. However, this was not the case because quantitative RT-PCR experiments using a SCN5A TaqMan probe did not reveal any difference between control and mdx5cv hearts (Figure 2C). Similarly, the mean mRNA level of K,2.1 was not modified in mdx5cv hearts. Thus, the decreased Na,1.5 content may be attributable to either a reduced translation rate or increased turnover of the channel protein in the absence of dystrophin.

**Sodium Current Is Decreased in mdx5cv Cardiomyocytes**

To investigate whether the decrease of the total cardiac Na,1.5 protein amount seen in mdx5cv hearts alters the cellular sodium current (I Na), we performed patch-clamp experiments using freshly isolated ventricular myocytes in the whole-cell configuration. Peak I Na density was reduced by 29±2% in mdx5cv myocytes compared with control cells (Figure 3A, 3B). This I Na reduction was not caused by alterations of the voltage-dependence of either steady-state activation or inac-
ECGs of mdx<sup>5cv</sup> Mice Reveal Conduction Defects

Subsequently, we investigated the consequences of this altered cellular excitability on surface ECGs of control and mdx<sup>5cv</sup> mice. Figure 4 shows representative ECG recordings (leads I, II, and III) of one control and two mdx<sup>5cv</sup> mice. The mdx<sup>5cv</sup> ECGs were characterized by a significant prolongation of the QRS complex (18±5%), a decrease of the P wave amplitude (6%), and a trend, albeit not significant, to prolonged P wave duration (Table 2). These findings point toward both intra-atrial and intra-ventricular conduction defects. Note that the heart rate and ventricular repolarization, reflected on the mouse ECG by the ST interval (similar, but not equivalent, to the JT interval in humans) were not different between mdx<sup>5cv</sup> and control mice (Table 2).

Mdx<sup>5cv</sup> Cardiac Tissues Do Not Show any Sign of Fibrosis or Cellular Hypertrophy

Because the intra-ventricular conduction defects may also have been caused by fibrosis of the mdx<sup>5cv</sup> ventricular myocardium, a phenomenon described in older mdx mice, we (H.A.L.) analyzed, in a blinded fashion, ventricular sections that were prepared with an elastic van Gieson stain. However, there was no increase in interstitial fibrosis neither in mdx<sup>5cv</sup> nor in control mice (n=4 animals of each group). In addition, we did not find any difference in nuclear and/or cytoplasmic size that may have reflected cardiac hypertrophy (not shown).

**Discussion**

The principal findings of this study are: (1) that cardiac dystrophin and syntrophin proteins are partners of the main cardiac voltage-gated sodium channel Nav1.5, and that this interaction depends exclusively on the C-terminal PDZ domain-binding motif of Nav1.5; (2) that Nav1.5 protein level is reduced in ventricles of dystrophin-deficient mdx<sup>5cv</sup> mice; and (3) that dystrophin-deficient mdx<sup>5cv</sup> myocardium shows a significant prolongation of the QRS complex and a decrease of the P wave amplitude, consistent with conduction defects.
(an animal model of DMD); and (3) that this reduced expression of Nav1.5 mdx<sup>5cv</sup> mice leads to decreased cellular sodium current and conduction defects that could be documented by surface ECGs.

### Description of the Protein Complex

Previous studies have already reported that Nav1.5 interacts with syntrophin proteins.<sup>4,5</sup> In the present study, we went one step further and characterized this complex by showing that this interaction with syntrophins depends largely on the PDZ domain-binding motif, and that a direct dystrophin-Nav1.5 interaction via the PY-motif of Nav1.5 was unlikely. We also translated this concept into the human situation by reproducing it on human heart protein extracts. Moreover, we obtained evidence suggesting that /H9251<sup>1</sup>- , /H9252<sup>1</sup>- , and /H9252<sup>2</sup>-syntrophins are likely to be the bona fide cardiac partners of Nav1.5, because, in analogy to findings of Iwata et al.,<sup>15</sup> /H9253-syntrophin proteins were not detected in cardiac lysates. Several other ion channels bearing a PDZ domain-binding motif have been reported to be part of the dystrophin protein complex (DPC) located at the plasma membrane of different cell types. Among them, the Kir2.x channels are expressed in cardiac cells,<sup>16</sup> Nav1.4<sup>4,17</sup> and aquaporin-4<sup>18</sup> are expressed in the skeletal muscle, and Kir4.1 has been localized to glial cells.<sup>19</sup> Our present studies have for the first time investigated pathological cardiac phenotypes related to altered function of these channels using the model of mdx mice.

### Colocalization of Dystrophin and Na<sub>1.5</sub>

Cardiac Na<sub>1.5</sub> channels are localized at the intercalated disk regions of cardiomyocytes,<sup>3,20</sup> as well as in the lateral membranes.<sup>3,21-23</sup> However, dystrophin has been shown to be absent from the intercalated discs of human<sup>24</sup> and rat<sup>25</sup> cardiac cells. These findings, in conjunction with the present results, suggest that at least two distinct pools of Na<sub>1.5</sub> channels co-exist in the plasma membrane of cardiac myo-
Is Decreased Expression Level of Channel Proteins a General Phenomenon?

Dystrophin is a large protein that exerts multiple roles. Its absence leads to a reduced expression of DPC proteins in skeletal muscle and cardiac tissue. In skeletal muscle of mdx mice, the expression of Na\(_{1.4}\) and aquaporin-4 are significantly reduced. In our animal model, a small reduction of K\(_{\text{r}}\)2.1 was also observed. The relevance of this phenome-
on is most likely marginal since no repolarization abnormality on the ECG was observed. In analogy to our present findings, the reduced protein expression was not attributable to decreased mRNA levels. In contrast, it should be noted that Na\(_{\text{r}}\) channel expression in spermatozoa has been shown to be increased when dystrophin is absent. Furthermore, in α1-syntrophin-deficient mice, the abundance of Na\(_{\text{r}}\) channels at the neuromuscular junction was not modified, whereas their localization was altered. Altogether, these findings suggest that one of the possible roles of dystrophin is to stabilize specific ion channel proteins by unknown mecha-
nisms (similarly to the other DPC proteins) in the plasma membrane. Whether dystrophin may be important for the trafficking, anchoring, regulation of ion channel protein turnover, or other phenomena remains to be further studied.

**ECG Alterations Are Consistent With Previous Studies**

In mdx\(^{5cv}\) mice, the QRS complex duration, reflecting intra-
ventricular conduction, was prolonged by 18%. These data compare to \(\approx 39\%\) increase in QRS duration in 10-week-old mice expressing only one SNC5A allele, where cellular Na\(_{\text{r}}\) is reduced to \(\approx 50\%\) of control mice. These QRS prolongation values are in good agreement with the estimate that can be inferred from the data of Shaw and Rudy where a reduction of I\(_{\text{Na}}\) by 29% (this study) or 50% would lead to a prolongation in duration for a given distance of \(\approx 18\%\) and \(\approx 33\%\), respectively. Furthermore, the intra-atrial conduction defects seen in mdx\(^{5cv}\) mice in our present study are consistent with corresponding alterations reported in the studies men-
tioned above. Because connexin-43 levels are not altered, and in the absence of fibrosis, we propose that the cardiac electrical phenotype of mdx\(^{5cv}\) mice is a “pure” conduction defect secondary to the reduction in Na\(_{1.5}\) protein level in absence of dystrophin. Whereas these considerations pertain to young (10 to 12-week old) mdx\(^{5cv}\) mice, it is well possible that older mice go onto develop a more complex cardiac phenotype with fibro-fatty degenerative features.

**Relevance for the Human Cardiac Dystrophinopathies**

DMD and Becker patients frequently show cardiac manifesta-
tions secondary to the absence of dystrophin in cardiac tissue. Many of these patients display dilated cardiomyopathy and heart failure. In addition, ECG abnormalities can also be detected in up to 60% of 10-year-old DMD patients, and

| TABLE 2. ECG Characteristics of Control and mdx\(^{5cv}\) Mice in Lead I |
|-----------------|----------|---------|----------|----------|----------|----------|----------|----------|
|                | n RR (ms) | Pdur (ms) | Pamp (µV) | PR (ms)  | QRS (ms) | QT (ms)  | QTc (ms) | ST (ms)  |
| Control        | 12 141±4 | 11.7±0.28 | 86±3     | 50.5±2.48 | 14.2±0.28 | 57.2±1.13 | 48.5±1.20 | 43.3±1.19 |
| mdx\(^{5cv}\)   | 13 139±4 | 12.4±0.37 | 70±5     | 47.2±1.43 | 16.7±0.69 | 58.3±1.11 | 49.6±1.12 | 41.6±0.84 |
| \(P\)          | ns       | ns       | <0.05    | ns       | <0.005   | ns       | ns       | ns       |

RR indicates RR interval duration; Pdur, P-wave duration; Pamp, P-wave amplitude; PR, PR interval duration, QRS, QRS-complex duration; QT, QT interval duration; QTc, corrected QT interval duration; n.s., not significant. Data are expressed as mean±SEM.
among those, conduction defects are frequent. Indeed, life-threatening arrhythmia can cause sudden death in these dystrophinopathy patients. The relevance of early functional defects involving Na,1.5 in mdxcv mice on the genesis of cardiac dysfunction in DMD patients is a matter of speculation. Two recent studies have reported that loss-of-function mutations in SCN5A may cause dilated cardiomyopathy in humans. Whether reduced Isn may lead to myocardial degeneration in dystrophinopathies, dilated cardiomyopathies, or the Brugada syndrome, as recently described, remains to be shown. In addition, based on the findings of this study, it could be proposed that genes encoding proteins of the DPC, may lead to congenital long QT syndrome, because it may be foreseen that absence or loss-of-function of syntrophins may also lead to a reduced syndrome, because it may be foreseen that absence or loss-of-function of syntrophins may also lead to a reduced function of Na,1.5. Interestingly, a recent abstract indicated that mutations in the caveolin-3 gene, a protein that is also part of the DPC, may lead to congenital long QT syndrome by altering the function of Na,1.5. This finding supports well the model of Na,1.5 being part of the dystrophin multiprotein complex.

Conclusions

In summary, our work provides evidence that at least one fraction of the Na,1.5 channels is part of the dystrophin complex in cardiomyocytes. Absence of dystrophin in young mdxcv mice leads to reduced Na,1.5 levels and hence reduced Isn associated with cardiac conduction defects. Whether these early functional defects may underlie the more severe morphological and mechanical alterations seen in dystrophinopathy patients warrants further investigation.

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Disclosures

None.

References


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The Cardiac Sodium Channel Na\textsubscript{v}1.5 is Regulated by a Multiprotein Complex Composed of Syntrophins and Dystrophin

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Supplementary Materials and Methods

Animals

Wild type C57BL/6 mice, used as control, were purchased at Janvier (Le Genest St Isle, France), and C57BL/6Ros-5Cv (mdx<sup>5cv</sup>) mice were initially provided by U. Ruegg (University of Geneva) in agreement with Jackson laboratories (Bar Harbor, Maine), before being raised in our department. Male mice at an age of 10-14 weeks were used in this study. In humans and mice the dystrophin gene is located on the X-chromosome. In the mdx<sup>5cv</sup> allele, the dystrophin mRNA contains a 53-bp deletion of sequences from exon 10<sup>1</sup>. As a result, these animals do not express dystrophin in any tissue. All animal procedures were performed in accordance with the Swiss laws.

Cardiac tissue samples

The experimental procedures were approved by the ethical commission on clinical research of the faculty of medicine of the University of Lausanne. Human right atrial sample was collected from patients in normal sinus rhythm undergoing coronary bypass surgery. The tissue was placed in ice cold PBS for transportation before being processed in our laboratory. Mice were anesthetized by intraperitoneal injection of 100 μl of pentobarbital (50 mg/ml) and 100 μl of heparin (Liquemin 5000 U.I./ml, Roche, Basel, Switzerland), and heart ventricles were excised, rinsed with ice cold PBS and frozen in liquid nitrogen. Fresh human atrial appendage or frozen mouse ventricle was transferred into lysis buffer (50 mM TRIS pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and Complete<sup>®</sup> protease inhibitor cocktail from Roche). Tissues were then homogenized using a Polytron and a Teflon homogenizer. Triton Tx-100 was added to a
final concentration of 1% and solubilization occurred by rotating for 1h at 4°C. The
soluble fraction from a subsequent 15-min centrifugation at 13,000 g (4°C) was used for
the experiments. In order to load each lane of the SDS-page with equivalent amounts of
total proteins, the protein concentration of each lysate was measured in triplicate by
Bradford assay using a BSA standard curve.

Mass spectrometry peptide analysis
SDS-PAGE gels were stained for 3 h with 50 ml of the SYPRO® dye (Molecular Probes,
Eugene, USA) and rinsed 3x10 min with 10% methanol, 7% acetic acid. The gel was
visualized on a UV-light transilluminator. The bands of interest were excised and sent to
the Protein Analysis Facility of Lausanne where they were submitted to trypsin digestion,
liquid chromatography and tandem mass spectrometry (LC-MS/MS analysis) as
described in (http://www.unil.ch/paf).

Pull-down assays
The cDNAs encoding the 66 last amino acids of Na\textsubscript{v}1.5 WT, Y1977A and S2014Stop
were cloned into pGEX-4T1 (Amersham Bioscience, Piscataway, USA). Expression of
GST-fusion proteins in E.coli cells was induced with 0.2 mM IPTG for 3 h at 30°C. Cells
were harvested by centrifugation and resuspended in lysis buffer (20 mM Tris pH 7.5,
250 mM NaCl, 0.5% NP40, 1 mM EDTA, 1 mM PMSF, 0.2 mg/ml DNASE I (Roche) and
0.2 mg/ml Lysozyme (Roche). Soluble fraction of a 15-min centrifugation at 13,000 g
(4°C) was rotated for 1 h in the presence of GSH-sepharose (Amersham Bioscience) at
4°C. GST-pull-down assays of soluble fractions of ventricular lysate were performed
using GSH-sepharose beads containing either GST or the GST fusion proteins.
Na$_{v}$1.5 function is regulated by dystrophin

Following 2 h rotation at 4°C and washing (50 mM TRIS pH 7.5, 150 mM NaCl, 1% Tx-100, 1 mM PMSF) bound proteins were detected by Western blot.

Western blot and antibodies

Western blotting conditions and the specificity of the polyclonal Na$_{v}$1.5 antibody (ASC-005, Alomone) has been previously described$^2$. A control Western blot using the peptide antigen showing the specificity of the signal is presented in the supplementary Figure. The polyclonal antibody K$_{ir}$2.1 (APC-026) was purchased from Alomone (Jerusalem, Israel). Polyclonal connexin-43 antibody (71-0700) was obtained from Zymed (San Fransisco, USA). Monoclonal dystrophin antibody (MANDYS8) was from SIGMA (Buchs, Switzerland). Monoclonal pan-syntrophin antibody (MA1-745) was purchased from Affinity Bioreagents (Golden, USA). Polyclonal α1-, β1- and β2-syntrophin antibodies were kindly provided by M. Schaub (University of Zurich), and γ1- and β 2-syntrophin antibodies were from Santa Cruz Biotechnology (Santa Cruz, USA). Polyclonal Ca$_{v}$1.2 antibody was a gift from J. Hell (University of Iowa), Na,K-ATPase antibody was a gift from K. Geering (University of Lausanne) and Nedd4-2 antibody was a gift from O. Staub (University of Lausanne).

TaqMan real-time RT-PCR

Total RNA was extracted from whole ventricles according to described protocol in$^2$. cDNA was synthesized from 1 µg of total RNA using the MU-MLV reverse transcriptase according to the manufacturer protocol (Q-Biogene EMMLV100, Irvine, USA). Fifty nanograms of cDNA combined with 1x TaqMan Universal Master Mix (Applied Biosystems, Foster, USA) and 1 µl of either Na$_{v}$1.5, K$_{ir}$2.1 or glyceraldehyde-3-
phosphate dehydrogenase (GAPDH) probe (Applied Biosystems respectively Mm99999915, Mm00434616 and Mm00451971) were loaded into each well. The 96 well thermal plate was cycled at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. GAPDH was used as reference gene for normalizing the data. The comparative threshold cycle relative quantification method was used to compare the amounts of mRNA in control and mdx mice. Samples were measured in duplicate.

**Isolation of cardiac myocytes and patch clamp experiments**

Adult mouse ventricular myocytes were isolated as described in2. Whole-cell configuration of the patch-clamp technique was used to record \(I_{Na}\). Experiments were performed at room temperature (22-23°C), using a VE-2 (Alembic Instruments) amplifier allowing the recording of large sodium currents2. Pipettes (tip resistance 1-2 MΩ) were filled with a solution containing 60 mM CsCl, 70 mM Cesium asparte, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM HEPES, 11 mM EGTA and 5 mM Na\(_2\)ATP (pH was adjusted to 7.2 with CsOH). Myocytes were bathed with a solution containing 10 mM NaCl, 120 mM NMDG-Cl, 2 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), 5 mM CsCl, 10 mM HEPES, and 5 mM Glucose (pH was adjusted to 7.4 with CsOH). Holding potentials were -120 mV and \(I_{Na}\) densities (pA/pF) were obtained by dividing the peak \(I_{Na}\) by the cell capacitance. Peak currents were measured during a current voltage protocol. No leak subtraction was performed; and the values were not corrected for the measured ~16 mV junction potential.

To quantify the voltage-dependence of steady-state activation and inactivation, data from individual cell were fitted with Boltzmann relationship, \(y(V_m) = 1/1 + \exp((V_m-V_{1/2})/K)\), in which \(y\) is the normalized current or conductance, \(V_{1/2}\) is the voltage at which half of
Na\textsubscript{v}1.5 function is regulated by dystrophin. The available channels is inactivated, \( k \) is the slope factor, and \( V_m \) is the membrane potential.

**ECG**

The mouse ECG was recorded as previously described\textsuperscript{3}. The software ECGAuto (EMKA Technologies, Paris, France) was used to analyze the data recorded. P-wave duration (\( P_{\text{dur}} \)) was measured from the first deflection of the P-wave to the point where it rejoined the isoelectric line. The latter was set at the first deflection of the P-wave. The PR interval was measured from the beginning of the P-wave to the beginning of the QRS complex. The QRS interval was measured from the first deflection of the Q-wave (or the R-wave when the Q-wave was absent) to the cross-point between the isoelectric line and the ST segment. The QT interval was measured from the beginning of the QRS complex to the end of the T-wave (\( T_{\text{end}} \)). \( T_{\text{end}} \) was defined as the point where the negative part of the T-wave returned to the isoelectric line. Corrected QT interval (QTc) was calculated according to Mitchell and coworkers\textsuperscript{4}:

\[
\text{QTc} = \frac{\text{QT}}{\sqrt{\frac{R_R}{100}}}
\]

**Histology and image analysis**

Four \( \mu \)M sections through the sub-valvular plane of formaldehyde-fixed paraffin-embedded hearts were prepared and stained with Elastica van Gieson stain according to standard protocols. Three representative 20x fields were photographed, using an Olympus digital camera C5050 and a standard diagnostic microscope, from the subendocardial, central, and subepicardial region of the left ventricle and imported into Photoshop (version 7.0, Adobe systems Inc.). Using the *magic wand* tool and the *select similar* command, all dark-stained nuclei were selected and their surface quantified by...
the histogram tool (given in pixel per inch, and then translated into µm² according to the total magnification of the image). The mean nuclei size was calculated by the division of the total nuclear area by the number of nuclei as counted using a commercially available plug-in (count marks, The image processing toolkit, Reindeer Games, Charlotte, USA.) and given in µm². In the absence of notable tissue fibrosis, edema, or prominent intra-myocardic vessels, the cytoplasmic area was calculated as the total area of the 20x field minus the nuclear area (see above) then divided by the number of nuclei assessed as described above.

**Statistical analyses**

Data are represented as mean values ± SEM. Two-tailed Student t-test was used to compare means. Statistical significance was set at P<0.05.
Supplementary figure. Control of the anti-Na\textsubscript{v}1.5 polyclonal rabbit serum (ASC-005, Alomone). (A) Western blot of lysates of non-transfected HEK293 cells (HEK), HEK293 cells transfected with Nav1.5 cDNA as described in\textsuperscript{5}, and ventricular lysates of WT (control) and mdx mice (as described in Materials and Methods). (B) Western blot of the same lysates as in (A) but with the primary antibody preincubated with the control peptide antigen (DRLPKSDSEDGPRALNQLSC, corresponding to residues 493-511 of mouse Nav1.5, 1 µg peptide/1 µg antibody).
References for the Online Data Supplement


