The cardiac voltage-gated sodium channel, Nav1.5, plays a central role in cardiac excitability and impulse propagation and associates with the dystrophin multiprotein complex at the lateral membrane of cardiomyocytes. It was previously shown that Nav1.5 protein content and the sodium current (I\textsubscript{Na}) were both decreased in cardiomyocytes of dystrophin-deficient mdx\textsuperscript{SCV} mice. In this study, wild-type and mdx\textsuperscript{SCV} mice were treated for 7 days with the proteasome inhibitor MG132 (10 μg/Kg/24 h) using implanted osmotic mini pumps. MG132 rescued both the total amount of Nav1.5 protein and I\textsubscript{Na} but, unlike in previous studies, the novo expression of dystrophin was not observed in skeletal or cardiac muscle. This study suggests that the reduced expression of Nav1.5 in dystrophin-deficient cells is dependent on proteasomal degradation.

Keywords: sodium channels, dystrophin, proteasome, proteasome inhibitors, MG132, electrophysiology
MATERIALS AND METHODS

ANIMALS

Wild-type (WT) C57BL/6 mice (Janvier, Le Genest St Isle, France), and C57BL/6Ros-TgSCV (mdx<sup>5cv</sup>) mice (Jackson laboratories, Bar Harbor, Maine) were raised at the department of pharmacology of the University of Lausanne. Male mice aged 12–16 weeks were used in this study. All animal procedures were performed in accordance

with Swiss and cantonal laws.

MINI PUMPS

Osmotic mini pumps (ALZET model 1007D, Alzet Osmotic Pump Company, Cupertino, USA) were implanted in the anterior back region of the mice. Pumps were filled up with either a MG132 solution or with the vehicle alone (0.9% NaCl), according to the ALZET filling procedure. MG132 (C2211, SIGMA, Buchs, Switzerland) was delivered at a dose of 10 μg/Kg/24 h. Two millimolars MG132 aliquot were added to dimethylsulfoxide (Merck, Darmstadt, Germany), before being further diluted to the appropriate concentration in 0.9% NaCl.

MICRO VENTRICULAR MYOCYTE ISOLATION

Seven days after implantation of the osmotic pump, the mice were heparinized with 100 μl of heparin (Liquemin 5000 IU/ml, Roche, Basel, Switzerland). They were then euthanized with an intraperitoneal injection of pentobarbital and subjected to collagenase retroperfusion. The procedure for mice ventricular myocyte isolation was previously described in detail (Gavillet et al., 2006). Approximately 10% of the isolated myocytes were plated on a laminin coated dish and used for patch clamp experiments; the remaining myocytes were frozen in pellet form. The frozen pellets were subsequently used for mRNA or protein extraction.

PROTEIN EXTRACTION

The gastrocnemius muscles were removed, washed with ice cold PBSx10 and frozen in liquid nitrogen. Frozen myocytes and skeletal muscle were transferred into lysis buffer (50 mM TRIS pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and Complete® protease inhibitor cocktail from Roche). Tissues were then homogenized using a Polytron. Triton X-100 was added to a final concentration of 1% and solubilization occurred by rotating for 1 h at 4°C. The soluble fraction obtained after 15 min of centrifugation at 13,000 x g was used for the experiments. In order to load each lane of the SDS-PAGE with equivalent amounts of total protein, the protein concentration of each lysate was measured in triplicate by Bradford assay using a BSA standard curve.

WESTERN BLOTS

The western blotting conditions have been previously described (Gavillet et al., 2006). The polyclonal dystrophin antibody (MANDYS8) and polyclonal actin antibody (ASC-005) were purchased from Alomone (Jerusalem, Israel). The monoclonal dystrophin antibody (MANDYS8) and polyclonal actin antibody (A2066) were obtained from SIGMA. The polyclonal Na<sub>V</sub>1.5 antibody (ASC-005) was purchased from Alomone (Jerusalem, Israel).

MICE VENTRICULAR MYOCYTE mRNA EXTRACTION

mRNA was extracted from frozen myocytes using the RNeasy Mini Kit, according to the manufacturer’s protocol (Qiagen, Hombrechtkikon, Switzerland). cDNA was synthesized from 1 μg of RNA using the M-MLV reverse transcriptase, according to the manufacturer’s protocol (Q-Biogene EMMLV 100, Irvine, USA). Fifty nanograms of cDNA combined with 1x TaqMan Universal Master Mix (Applied Biosystems, Foster, USA) and 1 μl of probe were loaded into each well. The SCN5A probe (Mm00451971), the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (Mm09999915), the SCN1B probe (Mm00441210) and the Nedd4-2 probe (Mm00459384) were obtained from Applied Biosystems. The 96 well thermal plate was cycled at 95°C for 2 min and 90°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. GAPDH was used as a reference gene to normalize the data. The comparative threshold cycle relative quantification method was used to compare the amounts of mRNA in control and mdx<sup>5cv</sup> mice. Samples were measured in duplicate.

PATCH CLAMP EXPERIMENTS

Only rod-shaped myocytes with distinct edges were selected for patch clamp experiments. The whole-cell configuration of the patch-clamp technique was used to record I<sub>Na</sub>. Experiments were performed at room temperature (22–23°C). Current recordings were performed using a VE-2 (Alembic Instruments) amplifier. Borosilicate glass pipettes (tip resistance 1–2 MΩ) were filled with a solution containing 60 mM CsCl, 70 mM cesium aspartate, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 11 mM EGTA (ethylene glycol tetracetic acid), and 3 mM Na<sub>2</sub>ATP (pH adjusted to 7.2 with CsOH). Myocytes were bathed with a solution containing 10 mM NaCl, 120 mM NMDG-Cl (N-methyl-D-glucamine chloride), 2 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, 5 mM Ca<sub>2</sub>SO<sub>4</sub>, 10 mM HEPES, and 5 mM glucose (pH adjusted to 7.4 with CsOH). Holding potentials were –120 mV and current densities (pA/pF) were obtained by dividing the peak I<sub>Na</sub> by the cell capacitance obtained using the transient capacitive current caused by a +5 mV pulse from the holding potential. Peak currents were measured during a current voltage protocol. To quantify the voltage-dependence of steady-state activation and inactivation, data from individual cells were fitted with the Boltzmann relationship, y(V<sub>m</sub>) = 1/1 + exp[(V<sub>m</sub> - V<sub>1/2</sub>)/k], in which y is the normalized current or conductance, V<sub>1/2</sub> is the voltage at which half of the available channels are inactivated, k is the slope factor, and V<sub>m</sub> is the membrane potential.

STATISTICAL ANALYSES

Data were represented as mean values ± SEM. Two-tailed Student’s t-test was used to compare means. Statistical significance was set at P < 0.05.

RESULTS

THE PROTEASOME INHIBITOR MG132 RESCUES NA<sub>V</sub>1.5 PROTEIN LEVELS AND THE SODIUM CURRENT IN mdx<sup>5cv</sup> MICE

The cardiac voltage-gated sodium channel, Na<sub>V</sub>1.5, is part of the DMC1 mouse cardiomyocytes (Gavillet et al., 2006). The Na<sub>V</sub>1.5...
protein content and the $I_{Na}$ were both decreased in mdx$^{cv}$ mice, in which dystrophin is not expressed (Gavillet et al., 2006). In addition, it was shown that the sodium channel could be ubiqui-
tylated by ubiquitin protein ligases of the Nedd4 family, thereby regulating the density of the channel at the cell membrane (van Bemmelen et al., 2004). In order to determine whether the ubiq-
uitin proteasome system is implicated in the diminution of the sodium channel in the cardiomyocytes of dystrophin-deficient mice, control and mdx$^{cv}$ mice were treated with the proteasome inhibitor MG132. Osmotic mini pumps were implanted subcuta-
nearly and delivered MG132 at a dose of 10 μg/kg/24 h over a 7-day period. Western blot experiments were performed using car-
diomyocyte lysates of mdx$^{cv}$ and control mice, both treated with either MG132 or saline solution (0.9% NaCl). The protein content of Na, 1.5 in the cardiomyocytes was quantified by digital density measurements of several Western blots, such as the one represented in Figure 1A. The total amount of Na, 1.5 protein was decreased by 49 ± 3% in the ventricular myocytes of mdx$^{cv}$ mice treated with the saline solution, as compared to control mice (Figures 1A,B). The MG132 treatment increased the protein level of Na, 1.5 in mdx$^{cv}$ cardiomyocytes to a level similar to that in control mice (Figures 1A,B). The proteasome inhibitor had no effect on the Na, 1.5 protein content in control mice (Figures 1A,B). Finally, Na, 1.5 mRNA quantification was performed using real time quan-
titative PCR. No significant difference of the Na, 1.5 transcript between mdx$^{cv}$ and control mice was observed in either treatment (Figure 1C).

The $I_{Na}$ was decreased by 29 ± 6% in mdx$^{cv}$ mice, as com-
pared to that in the controls (Figures 2A,B). The proteasome inhibitor had a strong effect on the $I_{Na}$ of mdx$^{cv}$ cardiac cells, increasing the current to a level similar to that found in control mice (Figures 2A,B). The effect of MG132 treatment on $I_{Na}$ was restricted to an increase in the current density, since nei-
ther the voltage-dependence of activation nor the steady state of inactivation were affected by the treatment (Figure 2C).

MG132 TREATMENT DOES NOT RESCUE DYSTROPHIN EXPRESSION IN SKELETAL OR CARDIAC MUSCLES
Bonuccelli et al. (2003) previously reported that the systemic treat-
ment with 10 μg/Kg/24 h of MG132 rescued the expression of
FIGURE 2 | Effects of MG132 treatment on the sodium current properties and mRNA level. (A) Current density-voltage relationship of $I_{Na}$ in control and mdx<sup>5cv</sup> mice treated with MG132 or 0.9% NaCl, as indicated. The protocol is indicated in inset. (B) Bar graph quantifying the amounts of sodium current in control and mdx<sup>5cv</sup> ventricular myocytes. Four cells were patched for each mouse and the number of mice used for quantification is indicated in the bars. The “normalized current” represents the maximum current density recorded at a given voltage ($-25$ mV). (C) Steady-state activation and inactivation curves. The protocol is indicated in inset. The number of mice used for quantification is indicated in the bars. Results are expressed as normalized mean signal intensity. *$P < 0.05$, n.s. not significant.

the dystrophin protein in skeletal muscle of the “original” mdx mouse strain. In the present study, Western blots of mdx<sup>5cv</sup> gastrocnemial muscle lysates were performed in order to determine whether dystrophin is expressed in skeletal muscle upon treatment with MG132. The dystrophin antibody used for the Western blots was directed against the actin binding site in the N-terminus. The mdx<sup>5cv</sup> mouse strain has a mutation in exon 10, which leads to a premature stop codon in the full-length transcript (Im et al., 1996). One can assume that if a shorter dystrophin form had been produced in mdx<sup>5cv</sup> muscles upon MG132 treatment, it may have been detected. As expected, dystrophin expression was undetectable in cardiac and skeletal muscle lysates of mdx<sup>5cv</sup> mice treated with 0.9% NaCl (Figures 3A,B). However, contrary to that described with the “original” mdx mice, MG132 treatment did not rescue the dystrophin expression in mdx<sup>5cv</sup> skeletal muscle or cardiomyocytes (Figures 3A,B).

Nedd4-2 AND THE β1-SUBUNIT mRNA AMOUNTS ARE NOT MODIFIED BY MG132 TREATMENT

Na<sub>1.5</sub> was shown to be regulated by the ubiquitin ligase protein Nedd4-2, which is expressed in the heart (van Bemmelen...
et al., 2004; Rougier et al., 2005). The β-subunits of Na$_v$1.5 were shown to modulate channel activity (Yu et al., 2005). In addition, the β1-subunit of Na$_v$1.5 (encoded by the gene SCN1B) was described to be down-regulated in the skeletal muscle of DMD patients (Haslett et al., 2002). In order to determine whether these proteins play a role in the regulation of Na$_v$1.5 in mdx$^{5cv}$ mice treated with MG132 or 0.9% NaCl, real time quantitative PCR experiments were performed to quantify the relative amounts of mRNA. Figures 4A,B illustrate that there are no differences between the different tested conditions, suggesting that these proteins are not likely involved in the modulation of Na$_v$1.5 upon MG132 treatment.

**DISCUSSION**

Treatment of “original” dystrophin-deficient mice with the proteasome inhibitor MG132 was shown to rescue dystrophin expression in their skeletal muscle (Bonuccelli et al., 2003). The authors did not, however, investigate the effect of MG132 on cardiac muscle (Bonuccelli et al., 2003). In the mdx$^{5cv}$ mouse strain, the Na$_v$1.5 protein content is decreased by ~50% and the $I_{Na}$ by ~30% (Gavillet et al., 2006). Studies using heterologous expression systems have demonstrated that ubiquitylation of Na$_v$1.5 could trigger its internalization and decrease $I_{Na}$ (van Bemmel et al., 2004). In the present work, control and mdx$^{5cv}$ mice were treated with MG132 in order to investigate the implications of the ubiquitin proteasome system on the regulation of Na$_v$1.5 in cardiac cells. The main findings of this study are: (1) the proteasome inhibitor MG132 rescues the sodium channel Na$_v$1.5 and $I_{Na}$ in mdx$^{5cv}$ cardiomyocytes, and (2) MG132 does not rescue the dystrophin expression in either cardiac or skeletal muscle in mdx$^{5cv}$ mice.

The proteasome is a proteolytic complex which rapidly degrades ubiquitylated proteins (Rock et al., 1994). MG132 is a molecule which reversibly blocks protein degradation by the proteasome (Rock et al., 1994). The results of the present work suggest that the decrease of Na$_v$1.5 observed in mdx$^{5cv}$ mice could be either directly or indirectly mediated by the proteasome. It is more likely that the proteasome is indirectly implicated in the regulation of Na$_v$1.5 since membrane proteins are primarily degraded by the lysosomal apparatus in eukaryotic cells, whereas the proteasome is involved in the proteolysis of cytosolic proteins (Lee and Goldberg, 1998). The activity of endocytic proteins is regulated by ubiquitin.
signals and the proteasome could control the degradation of these ubiquitylated proteins (Longra et al., 2006). Components of the endocytotic machinery that ubiquitylate membrane proteins, however, primarily monoubiquitylate and the proteasome recognizes polyubiquitylated proteins. It has been suggested that endocytotic proteins might be transiently polyubiquitylated and degraded by the proteasome (Salghetti et al., 2001). Altogether, these results suggest that the proteasome indirectly regulates Na\textsubscript{v} 1.5. Additional experiments using endocytotic or lysosome inhibitors should be carried out to help identify the proteolytic pathways involved in the degradation of Na\textsubscript{v} 1.5.

Unlike Bonuccelli et al. (2003), this study did not use the "original" mdx mouse strain which carries a premature stop codon in exon 23, since this strain was shown to have revertant fibers due to exon skipping events (Danko et al., 1992). This study used the mdx\texttextsuperscript{5cv} mouse strain which carries an A to T mutation in the middle of exon 10 that produces a new splice donor site and a premature stop codon in full-length transcripts (Im et al., 1996). MG132 treatment of mdx\texttextsuperscript{5cv} mice did not rescue dystrophin expression in skeletal or cardiac muscle. The different effects of MG132 treatment on the two mouse strains could be due to the nature of the dystrophin mutations. The mutation on the dystrophin gene of mdx\texttextsuperscript{5cv} mice may produce an unstable transcript which is not translated, whereas the "original" mdx strain may produce an unstable protein that accumulates upon MG132 treatment. This interpretation is supported by the study of Assereto et al. (2006) on the DMC composition of DMD and BMD muscle explants following in vitro treatment with 20 \mu M MG132. Only some of the DMD and BMD explants showed signs of DMC rescue after MG132 treatment, probably due to the nature of the dystrophin mutations.

In conclusion, it was observed that the proteasome inhibitor MG132 rescued the total amount of Na\textsubscript{v} 1.5 protein and the \(\text{J}_{\text{Na}}\) in cardiomyocytes, but did not rescue dystrophin expression in dystrophin-deficient mdx\texttextsuperscript{5cv} mice. Moreover, these results suggest that the proteasomal pathway is implicated in the degradation of Na\textsubscript{v} 1.5 channel in dystrophinopathies. We have yet to determine if the proteasome is directly or indirectly involved in the degradation of polyubiquitylated Na\textsubscript{v} 1.5 channel or if it regulates the endocytotic machinery which controls the density of the sodium channel at the plasma membrane. Additional experiments on the mechanisms of Na\textsubscript{v} 1.5 channel degradation and regulation in WT and dystrophin-deficient cardiac cells are needed to better understand the pathways involved in the maintenance of the Na\textsubscript{v} 1.5 channel in specific pools.

ACKNOWLEDGMENTS

We thank Dr. A. Felley and the members of the Hugers Abriel group for their useful comments on this manuscript. This research has received grants of the Swiss National Science Foundation to Hugers Abriel (310003_120707), Swiss Heart Foundation, Association Francaise contre les Myopathies (grant 14305).

REFERENCES


Rougier et al. MG132 rescues Na\textsubscript{v} 1.5 in mdx mouse.
Rougier et al. MG132 rescues Nav1.5 in mdx mice


Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 17 December 2012; accepted: 04 March 2013; published online: 26 March 2013.


This article was submitted to Frontiers in Cardiovascular Medicine, a specialty of Frontiers in Physiology.

Copyright © 2013 Rougier, Gavillet and Abriel. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.