Regulation of the cardiac voltage-gated Na^+ channel (H1) by the ubiquitin-protein ligase Nedd4

Hugues Abriel, Elena Kamynina, Jean-Daniel Horisberger, Olivier Staub*

Institute of Pharmacology and Toxicology, University of Lausanne, CH-1005 Lausanne, Switzerland

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Abstract The cardiac voltage-gated Na^+ channel H1, involved in the generation of cardiac action potential, contains a C-terminal PY motif (xPPxY). Since PY motifs are known ligands to WW domains, we investigated their role for H1 regulation and the possible involvement of the WW domain containing ubiquitin-protein ligase Nedd4, taking advantage of the *Xenopus* oocyte system. Mutation of the PY motif leads to higher peak currents when compared to wild-type channel. Moreover, co-expression of Nedd4 reduced the peak currents, whereas an enzymatically inactive Nedd4 mutant increased them, likely by competing with endogenous Nedd4. The effect of Nedd4 was not observed in the PY motif mutated channel or in the skeletal muscle voltage-gated Na^+ channel, which lacks a PY motif. We conclude that H1 may be regulated by Nedd4 depending on WW–PY interaction, and on an active ubiquitination site.

Key words: Voltage-gated Na^+ channel; PY motif; WW domain; Nedd4; Ubiquitination; Protein–protein interaction

1. Introduction

The cardiac isoform of the voltage-gated Na^+ channel is expressed in cardiomyocytes, where it plays a key role in the generation and propagation of the cardiac action potential, namely the phase 0 (upstroke phase) [1,2]. In the heart, the channel is thought to be composed of a main α subunit (260 kDa, gene H1 or SCN5A); the association of this α subunit with an auxiliary β1 subunit is still a matter of controversy [2,3]. The H1 gene is also expressed in embryonic and denervated skeletal muscle cells [4] and in specific brain regions, such as the limbic system [5]. The α subunit is the target of class I antiarrhythmic drugs [6] and has recently been shown to be mutated in three forms of congenital arrhythmic disorders: long QT syndrome [7], Brugada syndrome [8] and Lenègre-Lev syndrome [9]. It is comprised of four homologous domains, each containing six transmembrane and one P-loop domains, intra- and extracellular loops and cytosolic N- and C-termini [1]. As described by Einbond and Sudol [10], the C-terminus of the cardiac and brain voltage-gated Na^+ channel α subunits contains a conserved PY motif (consensus: PPxY, where x is any amino acid; see Fig. 1); such PY motifs are known to interact with WW domains [11] that are protein–protein interaction domains present in a growing number of different proteins [12].

We have recently described the function of an intracellular ubiquitin-protein ligase called Nedd4 (neuronal precursor cell expressed developmentally downregulated [13]). We have shown that Nedd4, which contains a C2 domain, three or four WW domains (depending on the species), and an ubiquitin-protein ligase HECT domain (homologous to E6-AP C-terminus [14]), was able to specifically interact via its WW domains with the PY motifs present on the amiloride-sensitive epithelial Na^+ channel (ENaC) [15]. Since ubiquitination of membrane proteins has been associated with their rapid internalization and degradation [16], we postulated that Nedd4 binds via its WW domains to the PY motifs of ENaC, and ubiquitinates the channel, which leads to its subsequent internalization and degradation. Indeed, we found that ENaC is a protein with a short half-life ($T_{1/2} \sim 1$ h) and is regulated by ubiquitination [17]. We further confirmed that Nedd4 acts as a negative regulator of ENaC, which upon binding to the ENaC PY motifs, controls the number of channels at the cell surface, likely by ubiquitination and subsequent endocytosis [18].

In view that both the voltage-gated Na^+ channel and Nedd4 are highly expressed in cardiac and nervous tissues, we wanted to know if the PY motif present on this channel plays a role in channel regulation and if such a role would involve Nedd4. We report here results supporting this hypothesis: (1) Expression of a PY motif mutated rat H1 Na^+ channel (rH1) in *Xenopus* oocytes increases its peak currents when compared with wild-type channel. (2) Overexpression of Nedd4 was able to decrease the peak currents of wild-type, but not of PY motif mutated channels. Our findings are consistent with the concept that endogenous Nedd4 negatively regulates this channel in *Xenopus* oocytes.

2. Materials and methods

2.1. Plasmids and constructs

Rat H1 (rH1; cardiac isoform of the α subunit) cloned into pSP64 and rat SKM1 (skeletal muscle isoform) [19,20] cloned into pBluescript SK^+ were a generous gift from Dr. L. Schild, University of Lausanne. Substitution of the tyrosine 1980 of rH1 by an alanine (Y1980A) was performed by PCR. Full-length wild-type and mutant (C938S) *Xenopus laevis* Nedd4 (xNedd4) cDNA were cloned into pSDeasy, as previously described [18].

2.2. Expression and function of rH1 and rSKM1 channels in Xenopus oocytes

rH1 and xNedd4 constructs were transcribed using SP6 RNA polymerase, and rSKM1 with T7 RNA polymerase. 12 ng cRNA encoding the α subunit with or without 2 ng cRNA encoding xNedd4 were co-injected into oocytes. Transient peak inward Na^+ currents (I_{Na}) were measured by the two-electrode voltage-clamp method after a 40 ms test pulse to −20 mV from a holding potential of −100 mV as previously described by Favre et al. [21]. Transient capacitive currents were compensated with a P/4 protocol. The perfused bath solution was (mM): 110 NaCl, 1.8 $CaCl_2$, and 10 mM HEPES–NaOH, pH

*Corresponding author. Fax: (41)-21-692-5355.
E-mail: olivier.staub@ipharm.unil.ch

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7.35. All values were normalized to the mean values in one given batch of oocytes. Data are presented as mean ± S.E.M. The statistical significance of the differences between the means was estimated using bilateral Student's *t*-test for unpaired data.

3. Results

3.1. Mutation of tyrosine 1980 in the PY motif of rH1 leads to an increase in I_{Na}

The presence of a conserved PY motif in the C-terminus of the cardiac voltage-gated Na^+ channel prompted us to investigate the possible role of this motif in the regulation of channel activity. We therefore substituted the tyrosine 1980, located in the PY motif, by an alanine (Fig. 1; asterisk). This tyrosine has been shown to be essential for the interaction with WW domains [11], moreover an analogous mutation in the epithelial Na^+ channel ENaC disrupts the interaction between ENaC and Nedd4 [15] and is linked to gain of function mutations causing Liddle's syndrome [22]. The wild-type and mutant rH1 α subunits were expressed in *Xenopus* oocytes and we measured the I_{Na} evoked by a 40 ms pulse from the holding potential (−100 mV) to −20 mV at different times after cRNA injection. The time course of I_{Na} increase was significantly different between the wild-type and the mutant channel. The peak Na^+ current of the Y1980A channel was about three times larger after 48 h expression when compared to the wild-type channel (Fig. 2) suggesting that the mutant Na^+ channel has a slower turnover at the surface membrane as observed with the corresponding Liddle's ENaC mutant channels [18].

3.2. The Y1980A mutation does not influence the voltage dependence of inactivation and activation

As the larger current measured with the mutant channel may be caused by a change in inactivation and/or activation voltage dependence, we tested these two parameters and found no difference between both constructs (Fig. 3). The fit of the inactivation relationship with the Boltzmann function of the wild-type and mutant yielded respectively a $V_{1/2}$ of −69.4 ± 0.9 mV and −68.6 ± 0.7 mV with slope factors of −4.6 ± 0.4 mV and −5.3 ± 0.3 mV. The activation curves (Fig. 3) were obtained by transforming the peak currents obtained at different potentials (V_m) into normalized conductance values with $G_{Na} = I_{Na}/(V_m - V_{rev})$ where V_{rev} is the reversal potential of the current–voltage relationship calculated from the experimental data points for each cell (average V_{rev} for wild-type = 56.1 ± 7.5 mV and for mutant = 56.2 ± 5.0 mV, $n = 8$). The Boltzmann fit of the activation curves for wild-type and mutant channels yielded respectively a $V_{1/2}$ of −30.8 ± 1.5 mV and −29.6 ± 0.8 mV with slope factors of 4.2 ± 0.5 mV and 3.9 ± 0.3 mV.

				*
r SCN5A/ r H1	*P15389*	1972	SSTSF	**PPSY**	DSVTRA
hSCN5A/ hH1	*Q14524*	1969	SSTSF	**PPSY**	DSVTRA
r SCN1A	*P04774*	1978	STAAC	**PPSY**	DRVTKP
r SCN2A	*P04775*	1967	PSTTS	**PPSY**	DSVTKP
r SCN3A	*P08104*	1913	SSTTS	**PPSY**	DSVTKP

Fig. 1. The PPXY motif is found in the cardiac and brain isoforms of the voltage-gated Na^+ channel. Presented are the amino acid sequences of the homologous and conserved C-terminus region in the cardiac (rat and human, rH1 and hH1) and the three brain (ratSCN 1A/2A/3A) isoforms of the Na^+ voltage-gated channel. Note the presence of the PPXY sequence (PY motif, bold in the gray box). All five sequences are from the Swissprot database (accession numbers in italic). The asterisk indicates the mutated tyrosine in rH1.

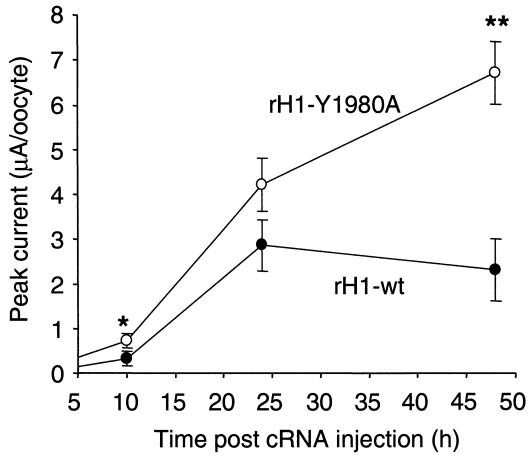

Fig. 2. The mutation Y1980A in the PY motif of the Na^+ channel (rH1) increases its peak current. The Y1980A mutation induced a significant increase (time dependent) of Na^+ channel peak current after injection of 12 ng cRNA of the wild-type and mutant α subunit in the oocytes. $n = 18$ oocytes from three different batches for each data point; $^*P < 0.05$, $^{**}P < 0.01$ vs. rH1 wild-type.

3.3. Overexpression of xNedd4 decrease the I_{Na} of the rH1 wild-type but not of the Y1980A mutant

In the case of the epithelial Na^+ channel ENaC, the PY motif is involved in the interaction with Nedd4. Because Nedd4 is also expressed in cardiac tissues [15], we asked whether Nedd4 plays a role in rH1 regulation. We co-injected into oocytes the cRNA encoding wild-type rH1 channels together with either the wild-type form of xNedd4 or a mutant

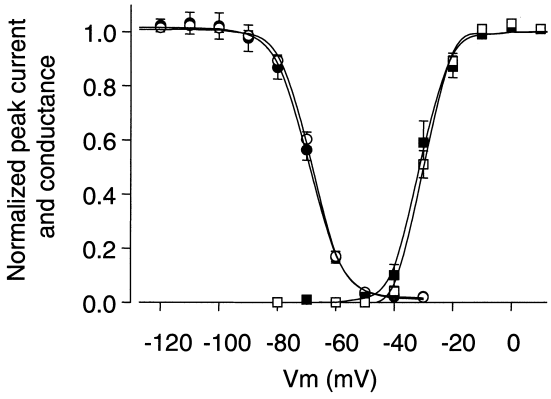

Fig. 3. The Y1980A mutation does not change the voltage dependence of the steady-state inactivation and activation. The steady-state inactivation relationships of wild-type (filled circles) and Y1980A mutant (empty circles) were studied by measuring the peak currents evoked by a 40 ms depolarization to −20 mV preceded by a conditioning pre-pulse of 460 ms at different (V_m) membrane potentials. The activation curves (wild-type filled squares and mutant empty squares) were obtained from the current–voltage relationships (40 ms pulses to the indicated test voltage) of individual cells as described in the text. The smooth lines are the best fitting curves corresponding to the equation I_{Na}/I_{max} or $G_{Na}/G_{max} = 1/(1+\exp[(V_m - V_{1/2})/k])$ where V_m is the conditioning or test potential, $V_{1/2}$ is the voltage for half maximal inactivation or activation, and k is a slope factor. No difference was noted between wild-type and mutant channels for both curves. $n = 8$ oocytes for each data point from two different batches.

bearing a Cys→Ser mutation (xN4C938S) at the conserved cysteine in the catalytic HECT domain. Such a mutation has been shown to abolish the enzymatic activity of HECT ubiquitin-protein ligases [14]. Moreover, in the *Xenopus* oocyte expression system, this mutant xNedd4 antagonizes the function of endogenous xNedd4 on ENaC [18]. Expression of rH1 alone (control) resulted in transient I_{Na} of about 2 μA/oocytes 15 h after cRNA injection (Fig. 4, left, first column). Upon co-expression of wild-type xNedd4 with rH1, I_{Na} fell to about 40% of the control value (Fig. 4, left, second column). In contrast, co-expression of the inactive Nedd4 mutant resulted in a significant increase of about 40% in I_{Na} relative to control oocytes (Fig. 4, left, third column). This finding suggests that Nedd4 negatively regulates the voltage-gated Na^+ channel.

3.4. The Nedd4-mediated negative regulation of rH1 is dependent on the presence of the PY motif

As Nedd4 has been shown to interact trough its WW domains with the PY motif of several proteins, we tested whether the negative functional regulation of rH1 by Nedd4 was dependent on the presence of the intact PY motif in the α subunit of the Na^+ channel. To this end, we expressed the mutant rH1 channel (Y1980A), which bears the disrupted PY motif. Fifteen hours after cRNA injection into oocytes, such a channel showed a comparable level of expression as compared to wild-type channels (Fig. 4, compare black columns). But in contrast to the wild-type channels, co-expression of neither the wild-type xNedd4 nor the catalytically inactive xN4C938S mutant did significantly change the Y1980A rH1 related peak currents (Fig. 4, right side), demonstrating that the PY motif is required for Nedd4-dependent regulation of rH1.

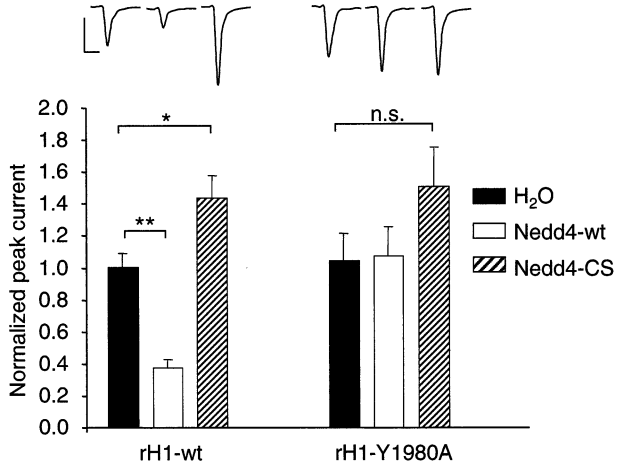

Fig. 4. Nedd4 negatively regulates the rH1 Na^+ channel, and this regulation is dependent on the PY motif of rH1. Oocytes were either injected with 12 ng cRNA of wild-type rH1 (left three columns) or rH1 channel with the Y1980A mutation (right three columns) together with either H_2O (black columns), wild-type xNedd4 (white columns, 2 ng cRNA) or xNedd4C938S (stripped columns, 2 ng cRNA). Currents were normalized to control mean values (wild-type channel; 1.7 ± 0.2 μA/oocyte). Twenty-four oocytes from four different batches were measured per condition. $*P < 0.05$ and $**P < 0.01$ represent levels of significance relative to conditions indicated by the square brackets. Inset: corresponding current traces (average of six oocytes of a given batch) for each studied condition; calibration: 5 ms and 1 μA.

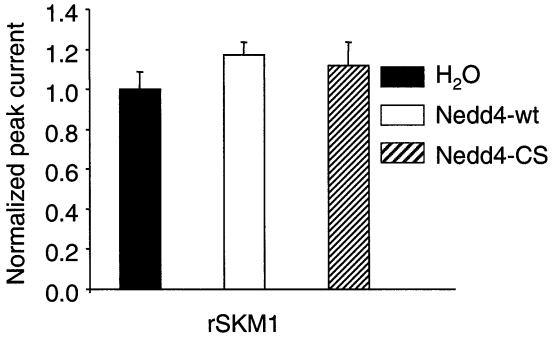

Fig. 5. Co-expression of Nedd4 does not influence the expression of rSKM1. Oocytes were injected with 12 ng cRNA of rSKM1 together with either H_2O (black columns), wild-type xNedd4 (white columns, 2 ng cRNA) or xNedd4C938S (stripped columns, 2 ng cRNA). Currents were normalized to control mean values (0.6 ± 0.1 μA/oocyte). Twenty oocytes from three different batches were measured per condition.

3.5. xNedd4 does not regulate the expression of the skeletal muscle SKM1 which does not bear a PY motif

The skeletal muscle isoform of the voltage-gated Na^+ channel (SKM1) shares 62% homology with the cardiac isoform (rH1), but in contrast to the cardiac and the three brain isoforms, SKM1 does not contain any PY motif [20]. In order to confirm that the observed negative regulation of rH1 was specific and dependent on the presence of the PY motif, we expressed the rat SKM1 (rSKM1) channel in oocytes together with wild-type and inactive mutant of xNedd4 and measured the peak I_{Na} as we did for rH1. Neither the co-expression of wild-type nor the mutant xNedd4 did influence the rSKM1 I_{Na} as shown in Fig. 5. Taken together our data show that Nedd4 regulates the cardiac voltage-gated Na^+ channel, in a manner that depends on the PY motif and on an active Nedd4 HECT domain.

4. Discussion

The goal of the present study was to investigate the functional role of the conserved PY motif present in the C-terminal tail of the cardiac voltage-gated Na^+ channel α subunit and a possible relation to the ubiquitin-protein ligase Nedd4. We observed that mutation of the key tyrosine residue of the PY motif increased channel related peak currents in a time-dependent manner. Moreover, co-expression of the rH1 channel with Nedd4 specifically decreased the measured Na^+ channel peak current of wild-type channels, whereas an inactive Nedd4 mutant (C938S) increased the peak currents, likely by competing against endogenous Nedd4. These effects on Na^+ channel activity were not observed with PY motif mutated channels or the skeletal muscle rSKM1, which lacks such a PY motif.

The results of this work present a striking analogy with the regulation of the epithelial Na^+ channel ENaC by Nedd4. Indeed, we and others have shown that the PY motifs of ENaC play an essential role in the control of ENaC activity. They act as binding sites for the Nedd4 WW domains [15,23], an interaction which likely promotes the ubiquitination of ENaC, its subsequent internalization and degradation [17,18]. The observation that the rH1 Y1980A mutant showed an increased activity is consistent with the concept that the PY motif is involved in the control of wild-type channel expres-

sion and/or membrane turnover, likely by interaction with endogenous Nedd4. This is further corroborated by the finding that overexpression of wild-type Nedd4 decreases channel activity, whereas an enzymatically inactive mutant (C938S) is able to stimulate it.

The observation that an active ubiquitin-protein ligase (HECT) domain is essential for suppression of channel activity suggests that an ubiquitination event is required for Nedd4-dependent action. Since Nedd4 seems to bind directly the channel, it is likely that either the α subunit itself or an associated protein becomes ubiquitinated. In the case of ENaC, we have demonstrated that the protein is ubiquitinated and that Nedd4 controls its surface expression. This remains to be shown for the voltage-gated Na^+ channel. The observation that mutation of the PY motif does not affect the activation or inactivation of voltage dependence suggests that the voltage-dependent gating properties are not altered. Moreover, an effect on the single channel conductance is not expected with mutations in this part of the channel. Supporting this notion, this region (the last 150 amino acids of the C-terminus) has recently been studied by chimerical studies between the H1 and SKM1 genes expressed in HEK 293 cells [24]. This work did not report any significant function for this portion of the channel, but on the other hand, it does not mention the absolute values of measured peak currents.

For ENaC naturally occurring mutations have been found, which either delete or mutate the PY motif, thereby causing Liddle's syndrome, an inherited form of human hypertension. These mutations lead to a gain of ENaC function. No similar mutations have been described so far for the voltage-gated Na^+ channel; this may be either due to a less obvious phenotype or in contrary, they may be lethal. Nevertheless it may be worth to search for such mutations in the voltage-gated Na^+ channel, mainly in disorders which are associated with gain of function of the voltage-gated Na^+ channel such as observed with one of the mutations causing the long QT syndrome (type 3), namely the deletion of KPQ 1507–1509 [25].

The relevance of the proposed regulatory mechanism of rH1 by Nedd4 is supported by the fact that Nedd4 is strongly expressed in the heart and the nervous tissues [15]. In neurons, the three brain specific Na^+ channel isoforms bear the PY motif (Fig. 1) suggesting that in these tissues, too, the same regulatory mechanism may take place. Moreover the H1 gene has recently been shown to be expressed in a very specific pattern in the central nervous system [5]. In contrast, the adult skeletal muscles may be regulated by other mechanisms, as it does not contain a PY motif and hence is probably not controlled by Nedd4.

In conclusion, based on our co-expression study in a heterologous expression system, we propose that the membrane turnover of the cardiac α isoform of the voltage-gated channel is regulated by the ubiquitin-protein ligase Nedd4. In addition, this mechanism may also take place in the nervous tissues with the brain isoforms of the Na^+ channel. In order to understand the molecular basis of the observed functional effects, the direct interaction between H1 and Nedd4 and the ubiquitination of the Na^+ channel remain to be demonstrated in future studies.

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