Regulation of Neuronal Voltage-gated Sodium Channels by the Ubiquitin-Protein Ligases Nedd4 and Nedd4-2*

Received for publication, March 12, 2004, and in revised form, April 13, 2004
Published, JBC Papers in Press, April 27, 2004, DOI 10.1074/jbc.M402820200

Andrew B. Fotiadis, Jenny Ekberg, David J. Adams, David I. Cook, Philip Poronnik, and Sharad Kumar

Nedd4 and Nedd4-2 are ubiquitin-protein ligases known to regulate a number of membrane proteins including receptors and ion transporters. Regulation of the epithelial Na⁺ channel by Nedd4 and Nedd4-2 is mediated via interactions between the PY motifs of the epithelial sodium channel subunits and the Nedd4/Nedd4-2 WW domains. This example serves as a model for the regulation of other PY motif-containing ion channels by Nedd4 and Nedd4-2. We found that the carboxyl termini of the six voltage-gated Na⁺ (Nav) channels contain typical PY motifs (PPXXY), and a further Na⁺ channel contains a PY motif variant (LXXPLPY). Not only did we demonstrate by Far-Western analysis that Nedd4 and Nedd4-2 interact with the PY motif-containing Na⁺ channels, but we also showed that these channels have conserved WW domain binding specificity. We further showed that the carboxyl termini fusion proteins of one central nervous system and one peripheral nervous system-derived Na⁺ channel (Na₁.2 and Na₁.17, respectively) are readily ubiquitinated by Nedd4-2. In Xenopus oocytes, Nedd4-2 strongly inhibited the activities of all three Na⁺ subunits (Na₁.2, Na₁.7, and Na₁.8) tested. Interestingly, Nedd4 suppressed the activity of Na₁.2 and Na₁.17 but was a poor inhibitor of Na₁.8. Our results provide evidence that Nedd4 and Nedd4-2 are likely to be key regulators of specific neuronal Na⁺ channel activity in vivo.

Voltage-gated sodium channels (Nav) are essential for the generation and propagation of action potentials in electrically excitable cells. These channels mediate the influx of Na⁺ ions in response to local depolarizing stimuli and thus play key roles in regulating excitation, secretion, and contraction (1). Na⁺ channel activity is crucial for the plasticity and development of the nervous system and the maintenance of excitability following nerve and tissue injury (1). Na⁺ channels are composed of a pore-forming α-subunit (~260 kDa) associated with one or more auxiliary β-subunits (~35 kDa) (1). The α-subunits are able to form functional channels, whereas the β-subunits modulate the biophysical properties of the α-subunit (2). Nine mammalian Na⁺ channel α-subunit genes have been characterized with distinct tissue-specific expression and electrophysiological properties (3). Based on expression profiles, individual Nav channel genes can be divided into several different groups. Nav₁.1, Nav₁.2, Nav₁.3, and Nav₁.6 are primarily found in the central nervous system; Nav₁.7, Nav₁.8, and Nav₁.9 are predominant in the peripheral nervous system, and skeletal and cardiac muscle cells express Nav₁.4 and Nav₁.5, respectively (3). The expression profile of the Nav channel types in excitable cells contributes to the specificity of their transduction and excitability properties (1). The importance of normal Na⁺ channel activity is evident from hereditary channel mutations that result in epilepsy, long QT syndrome, Brugada syndrome, and many other diseases (2).

Although it is known that trafficking and recycling is critical for the regulation of numerous ion channels, little is known about the regulation of Nav channels by this mechanism. It is well established that members of the Nedd4-family of ubiquitin-protein ligases (4–6) interact with specific WW domains of Nedd4 proteins (7). Not only do Nedd4 and Nedd4-2 interact with the PY motif-containing Na⁺ channels, but we also showed that these channels have conserved WW domain binding specificity. We further showed that the carboxyl termini fusion proteins of one central nervous system and one peripheral nervous system-derived Na⁺ channel (Na₁.2 and Na₁.17, respectively) are readily ubiquitinated by Nedd4-2. In Xenopus oocytes, Nedd4-2 strongly inhibited the activities of all three Nav subunits (Nav₁.2, Nav₁.7, and Nav₁.8) tested. Interestingly, Nedd4 suppressed the activity of Nav₁.2 and Nav₁.17 but was a poor inhibitor of Nav₁.8. Our results provide evidence that Nedd4 and Nedd4-2 are likely to be key regulators of specific neuronal Na⁺ channel activity in vivo.

This paper is available online at http://www.jbc.org

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
found to be ubiquitinated in vitro by Nedd4-2. When expressed in Xenopus oocytes with Na\textsubscript{\alpha} channel \textalpha-subunits, Nedd4 and Nedd4-2 were found to down-regulate the depolarization-activated Na\textsuperscript{+} currents.

**MATERIALS AND METHODS**

**Bacterial Expression Plasmids**—The Na\textsubscript{\alpha} channel carboxyl-terminal region-glutathione S-transferase (GST) fusion constructs were generated as follows. The carboxyl termini were PCR-amplified from mouse brain cDNA (Na\textsubscript{\alpha}1.1, Na\textsubscript{\alpha}1.2, Na\textsubscript{\alpha}1.3, and Na\textsubscript{\alpha}1.6), mouse day 18 embryo cDNA (Na\textsubscript{\alpha}1.5 and Na\textsubscript{\alpha}1.7), and mouse dorsal root ganglion cDNA (Na\textsubscript{\alpha}1.8) (donated by A. Stukowski) and cloned into either the BamHI or BamHI/EcoRI sites of pGEX-2TK (Amersham Biosciences). The number of carboxyl-terminal residues present in the various Na\textsubscript{\alpha}–GST fusion proteins are as follows: Na\textsubscript{\alpha}1.1, 64; Na\textsubscript{\alpha}1.2, 66; Na\textsubscript{\alpha}1.3, 62; Na\textsubscript{\alpha}1.5, 72; Na\textsubscript{\alpha}1.6, 57; Na\textsubscript{\alpha}1.7, 64; and Na\textsubscript{\alpha}1.8, 81. A construct containing mouse Nedd4-2 (\triangle C2,WW1,WW2) was generated by PCR amplification from the mouse plasmid pGEX-2TK cloned following into the EcoRI site of pGEX-2TK. A catalytically inactive Cys mutant of Nedd4-2 (Nedd4-2 cDNA followed by cloning into the EcoRI site of pGEX-2TK (Amersham Biosciences). The number of carboxyl-terminal residues present in the various Na\textsubscript{\alpha}–GST fusion proteins are as follows: Na\textsubscript{\alpha}1.1, 64; Na\textsubscript{\alpha}1.2, 66; Na\textsubscript{\alpha}1.3, 62; Na\textsubscript{\alpha}1.5, 72; Na\textsubscript{\alpha}1.6, 57; Na\textsubscript{\alpha}1.7, 64; and Na\textsubscript{\alpha}1.8, 81. A construct containing mouse Nedd4 and Nedd4-2 WW domains and carboxyl termini of \alpha, \beta, and \gamma ENaC in pGEX-2TK have been described previously (10, 12, 13). The construct containing mouse Nedd4 (amino acids 52–77) in pGEX was a gift of J.-P. Jensen and A. M. Weissman (NCI, National Institutes of Health).

**Proteins of GST Fusion Proteins**—Overnight cultures of Escherichia coli BL21 start (DE3)pLysS (Invitrogen) harboring the Nedd4-2 (\triangle C2,WW1,WW2) or Nedd4-2 (\triangle C2,WW1,WW2) Cys mutant GST expression plasmids were diluted 1:25, grown to log phase at 22 °C, and then induced with 1 mM isopropyl-1-thio-\beta-D-galactoside at 22 °C. All other GST fusion proteins were grown similarly but at 37 °C. Following induction, GST fusion proteins were purified as described previously (10, 12, 13). The protein concentrations were measured using a BCA kit (Pierce) and Coomassie Blue staining following separation by SDS-PAGE.

**SDS-PAGE and Far-Western Analysis**—\textsuperscript{32}P-labeled protein probes were produced by directly labeling the appropriate GST fusion protein using protein kinase A (New England Biolabs) as described previously (10). For Far-Western analysis, ~2 µg of each GST fusion protein was resolved by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (PerkinElmer Life Sciences). Membranes were blocked in Hyb75 and then hybridized with the appropriate \textsuperscript{32}P-labeled protein probes for 4 h at 4 °C in Hyb75 as described (15). Membranes were washed four times in Hyb75 and visualized by autoradiography. Band intensities were analyzed semi-quantitatively using ImageQuant Version 5.2 software (Molecular Dynamics). Far-Western binding intensities were measured against background probe hybridization levels except for bands representing the wild type individual WW domains, which were measured relative to the nonspecific binding of the corresponding mutant WW domains. To normalize binding intensities within individual Far-Western blots, these values were divided by the intensity of corresponding Coomassie Blue-stained protein bands and expressed as arbitrary units.

**In Vitro Ubiquitation Assays**—Carboxyl-terminal Na\textsubscript{\alpha}1.2 and Na\textsubscript{\alpha}1.7 GST fusion proteins and GST alone were \textsuperscript{35}S-labeled by using protein kinase A as described above. \textsuperscript{35}S-labeled proteins were incubated with 750 ng of purified Nedd4-2 (amino acids 52–777), GST, Nedd4-2 (\triangle C2,WW1,WW2), or the Nedd4-2-\triangle C2,WW1,WW2) Cys mutant in the presence of rabbit E1 (150 ng) and UbcH5b (300 ng) (both from Boston Biochem Inc.) in 25 mM Tris-HCl, pH 7.5, 120 mM NaCl, 2 mM MgCl\textsubscript{2}, 0.3 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 5 mM HEPES, 5 mM pyruvic acid, and 50 µg/ml gentamicin, pH 7.5) prior to recording. Three days after cRNA injection, whole cell Na\textsubscript{\alpha} channel currents were recorded from oocytes using the two-electrode (virtual ground circuit) voltage clamp technique. Micro-electrodes were filled with 3 M KCl and typically had resistances of 0.3–1.5 megohms. All recordings were made at room temperature (20–23 °C) using bath solution containing 100 mM NaCl, 2 mM KCl, 1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 3 mM HEPES, and 20 mM Hapes, pH 7.5, with NaOH. During recording, oocytes were perfused continuously at a rate of ~1.5 m/min. Using a GeneClamp 500B amplifier and pCLAMP 8 software (Axon Instruments Inc, Union City, CA), data were low pass filtered at 1 kHz, digitized at 10 kHz, and leak-subtracted on-line using a P-8 protocol and analyzed off-line. Initially, inward Na\textsuperscript{+} currents were generated by holding the cells at −70 mV and applying step depolarizations to membrane potentials from −50 mV to +50 mV. Inward Na\textsuperscript{+} currents were evoked with 100-ms depolarizing pulses at 10-s intervals to +20 mV (Na\textsubscript{\alpha}1.8) and 0 mV (Na\textsubscript{\alpha}1.7 and Na\textsubscript{\alpha}1.2) from a holding potential of −70 mV.

**RESULTS**

Several Na\textsubscript{\alpha} Channels Contain PY motifs in Their Carboxyl-terminal Regions—We analyzed amino acid sequences of mouse Na\textsubscript{\alpha} channel \textalpha-subunits to identify PY motif-containing channels. Conserved PY motifs conforming to the PXXY consensus were identified in both human and mouse Na\textsubscript{\alpha}1.1, Na\textsubscript{\alpha}1.2, Na\textsubscript{\alpha}1.3, Na\textsubscript{\alpha}1.5, Na\textsubscript{\alpha}1.7, and Na\textsubscript{\alpha}1.8. Na\textsubscript{\alpha}1.6 was found to contain a PY motif variant (LPY) that has been shown to act as a ligand for Nedd4 WW domains (16) and represents a previously unidentified PY motif-containing Na\textsubscript{\alpha} channel. Na\textsubscript{\alpha}1.4 and Na\textsubscript{\alpha}1.9 do not contain PY motifs. An alignment of Na\textsubscript{\alpha}1.1, Na\textsubscript{\alpha}1.2, Na\textsubscript{\alpha}1.3, Na\textsubscript{\alpha}1.5, Na\textsubscript{\alpha}1.6, Na\textsubscript{\alpha}1.7, Na\textsubscript{\alpha}1.8, and Na\textsubscript{\alpha}1.9 with Na\textsubscript{\alpha} channel Greek symbols is shown in Table I.
channel PY motif regions revealed a high degree of homology (Fig. 1A), suggesting that they may share similar WW domain binding specificity. Outside the PY motif itself, the homology between the PY motif-containing regions of the three ENaC subunits and the various Nav/H11001 sub-units was limited (Fig. 1A).

**Nedd4 and Nedd4-2 WW Domains Interact With the Carboxyl Termini of Seven Nav Channels**—To investigate whether interactions occur between PY motif-containing Na v channels and Nedd4 or Nedd4-2 WW domains, a Far-Western approach was employed. The carboxyl termini of PY motif-containing Na v channels and Nedd4 or Nedd4-2 WW domains, a Far-Western approach was employed. The carboxyl termini of PY motif-containing Na v channels were cloned from mouse cDNA and expressed as GST fusion proteins. Equal amounts of affinity-purified GST-Na v proteins were electrophoresed on SDS-PAGE, immobilized on PVDF membranes, and then probed with [32P]-labeled Nedd4 or Nedd4-2 proteins containing their entire WW domain regions. Nedd4 and Nedd4-2 proteins interacted with all of the seven Na v proteins tested to varying degrees but not with GST alone (Fig. 1B). Differences in affinity toward individual Na v proteins were similar for both Nedd4 and Nedd4-2 probes; in each case the binding of Na v 1.1, Na v 1.6, or Na v 1.7 was 30–50% weaker than that of the other Na v proteins (Fig. 1B). This was reproducible and was verified by semi-quantitative binding analysis.2 As expected, both Nedd4 and Nedd4-2 WW domain-GST fusion proteins bound the PY motif containing ENaC subunits. It has been suggested that an extended PY motif containing a leucine residue three positions carboxyl to the tyrosine residue (PPXYXXL) is a requirement for ENaC subunits to interact properly with Nedd4 WW domains (17). In the present work, we found that Nedd4 and Nedd4-2 WW domains interact with Na v PY motifs that each have a valine residue in this position. This discovery, taken together with the finding that Na v 1.6, which has an LPXY motif (Fig. 1A), can bind Nedd4 and Nedd4-2 WW domains suggests that an extended consensus sequence of (L/P)PXYXXL/V in Na v channels is recognized by Nedd4 family WW domains.

**The Na v Channel PY Motifs Have Conserved WW Domain Binding Specificity**—Previous studies have shown that interactions between ENaC and Nedd4 or Nedd4-2 are mediated by specific WW domains (10, 13). WW2 and WW3 of mouse Nedd4 (WW2, WW3, and WW4 of human Nedd4) and WW3 and WW4 of Nedd4-2 interact with each ENaC subunit in a PY motif-WW domain-specific manner in vitro (10, 13). To test which Nedd4 and Nedd4-2 WW domains mediate interactions with Na v proteins, GST fusion proteins containing wild-type and mutated individual WW domains of mouse Nedd4 and Nedd4-2 were generated. Equal amounts of the affinity-purified GST-WW domain proteins were immobilized on PVDF membranes after separation by SDS-PAGE and then probed with [32P]-labeled Na v protein probes as indicated on right-hand side of the gels. Molecular mass markers in kilodaltons are indicated on the left-hand side of gels.

---

2 A. B. Fotia and S. Kumar, unpublished data.
The regulation of \( \text{Nav} \) channels by Nedd4 and Nedd4-2 is not restricted to the \( \text{Nav} \) proteins tested but is also seen with ENaC subunits (10, 13), suggesting a strong conservation of mechanism for Nedd4- and Nedd4-2-mediated regulation. Because of the inherent difficulties in expressing full-length \( \text{Nav} \) channel \( \alpha \)-subunits, attempts to produce larger \( \text{Nav} \) proteins containing additional lysine-rich regions for ubiquitination assays were unsuccessful.

**Effects of Nedd4, Nedd4-2, and Nedd4/Nedd4-2 Cys mutants on peak \( \text{Na}^+ \) current amplitude.** Bar graphs represent the percent changes in peak \( \text{Na}^+ \) current amplitude relative to the control (mean \pm S.E. of 20–30 oocytes from 4–6 separate batches).

---

**Fig. 3.** Nedd4-2 ubiquitinates GST-\( \text{Nav} \)1,2 and GST-\( \text{Nav} \)1,7 carboxyl termini in vitro. \( ^{32}\text{P} \)-labeled \( \text{Na} \),1,2 and \( \text{NA} \),1,7 carboxy-terminal GST fusion proteins were incubated in reactions containing E1, E2 (UbcH5b), ubiquitin, and ATP with or without Nedd4, GST, Nedd4-2, or the Nedd4-2 Cys mutant (mut), as indicated, followed by SDS-PAGE. In the presence of Nedd4-2, higher molecular weight bands (indicated) accompany \( \text{Na} \),1,2 and \( \text{Na} \),1,7 proteins representing ubiquitinated species. In the presence of Nedd4, at least one higher molecular weight band (arrow) is observed above the \( \text{Na} \),1,2 and \( \text{Na} \),1,7 proteins. No ubiquitination of the \( \text{Na} \),1,2 or \( \text{Na} \),1,7 channel occurs when GST or the Nedd4-2 Cys mutant are used in place of Nedd4 or Nedd4-2, nor does ubiquitination of GST alone occur under any condition.

**Fig. 4.** Effects of Nedd4, Nedd4-2, and Nedd4/Nedd4-2 Cys mutants on peak \( \text{Na}^+ \) current amplitude.

---

The regulation of \( \text{Nav} \) channels by Nedd4 and Nedd4-2 was at least 2-fold stronger than to Nedd4 WW domains, whereas binding differences were less pronounced for Nav1,1, Nav1,2, and Nav1,3 (data not shown).

Strikingly, all seven \( \text{Nav} \) proteins were repeatedly found to bind the same WW domains, namely WW2 and WW3 of mouse Nedd4 and WW3 and WW4 of Nedd4-2, but did not bind the inactive mutants of each of these domains (Fig. 2). This binding pattern is not restricted to the \( \text{Nav} \) proteins tested but is also seen with ENaC subunits (10, 13), suggesting a strong conservation of mechanism for Nedd4- and Nedd4-2-mediated regulation of sodium and perhaps other ion channels. Further analysis of the binding data showed that the Nedd4 WW2 domain binding was slightly stronger than the Nedd4 WW3 binding with all \( \text{Nav} \) channel \( \alpha \)-subunits except \( \text{Nav} \),1,6, which showed stronger binding to WW3. The Nedd4-2 WW3 and WW4 domains bound to essentially the same degree with \( \text{Nav} \),1,1, \( \text{Nav} \),1,2, and \( \text{Nav} \),1,3; however, for \( \text{Nav} \),1,5, \( \text{Nav} \),1,6, \( \text{Nav} \),1,7, and \( \text{Nav} \),1,8 the Nedd4-2 WW3 domain showed 20–45% stronger binding than did the Nedd4 WW4 domain (data not shown).

**Nedd4-2 Ubiquitinates \( \text{Nav} \),1,2 and \( \text{Nav} \),1,7 Carboxyl Termini in Vitro**—The regulation of \( \text{Na} \) channels by Nedd4 and Nedd4-2 potentially occurs by ubiquitination followed by degradation. PY motif-containing \( \text{Nav} \) channel \( \alpha \)-subunits are generally lysine-rich at their intracellular carboxyl termini, indicating possible targets for ubiquitin conjugation. An in vitro ubiquitination assay was used to test this possibility. We incubated \( ^{32}\text{P} \)-labeled carboxy-terminal GST fusions of \( \text{Na} \),1,2, a central nervous system-specific channel, and \( \text{Na} \),1,7, a peripheral nervous system-specific channel, with catalytically active recombinant Nedd4 or Nedd4-2 proteins in a ubiquitination buffer. Because of difficulties in expressing full-length Nedd4-2, a truncated form of protein containing the WW3, WW4, and HECT domains was used. This protein contains the ligand (\( \text{Nav} \) and ENaC) and E2 binding regions and the catalytic domain. In the presence of Nedd4-2, higher molecular mass bands representing ubiquitinated forms were observed for both \( \text{Nav} \) proteins (Fig. 3). However, in the presence of Nedd4 the ubiquitinated forms of \( \text{Nav} \),1,2 and \( \text{Nav} \),1,7 were barely detectable (Fig. 3). GST alone or a mutated Nedd4-2 protein lacking ubiquitin-protein ligase activity had no effect (Fig. 3). These data indicate that Nedd4-2-mediated regulation of \( \text{Na} \) currents may occur via ubiquitination at the intracellular carboxyl-terminal region. Given that Nedd4 had only a weak ubiquitination effect on \( \text{Na} \),1,2 or \( \text{Na} \),1,7, Nedd4-mediated regulation of \( \text{Na} \) channels may occur by either ubiquitination elsewhere within the \( \text{Nav} \) channel \( \alpha \)-subunit or by some other mechanism. In separate assays with a known Nedd4 substrate, purified Nedd4 and Nedd4-2 proteins had equivalent ubiquitin-protein ligase activities. Other lysine-rich regions exist in some \( \text{Na} \) channels, which may serve as targets of ubiquitination by Nedd4. Because of the inherent difficulties in expressing full-length \( \text{Nav} \) channel \( \alpha \)-subunits, attempts to produce larger \( \text{Nav} \) proteins containing additional lysine-rich regions for ubiquitination assays were unsuccessful.

**Effects of Nedd4-2 on \( \text{Nav} \) Channel \( \alpha \)-Subunit-mediated \( \text{Na}^+ \) Currents in Oocytes**—Xenopus oocytes were used to investigate the effects of Nedd4 and Nedd4-2 on depolarization-activated \( \text{Na}^+ \) currents mediated by representative central nervous system and peripheral nervous system \( \text{Na} \) channels. Control oocytes expressed only the cRNA for the corresponding \( \text{Na} \) \( \alpha \)-subunits. We observed that the expression of Nedd4 had differential effects on the \( \text{Na}^+ \) currents mediated by the various \( \text{Na} \) channel \( \alpha \)-subunits. Nedd4 had no effect on the peak \( \text{Na}^+ \) current amplitudes mediated by \( \text{Na} \),1,8 (105.4 ± 10.7%; \( n = 20 \); Figs. 4 and 5). Increasing the amount of Nedd4 cRNA injected per oocyte to 50 ng also had no effect on \( \text{Na}^+ \) current amplitude (data not shown). However, when Nedd4 was co-expressed with \( \text{Nav} \),1,7, we observed a significant reduction in the peak \( \text{Na}^+ \) current amplitudes to 35.8 ± 6.2% (\( n = 20 \); \( p < 0.001 \)) of control current. Furthermore, Nedd4 had an equally pronounced effect on \( \text{Na} \),1,2, reducing the \( \text{Na}^+ \) current amplitude to 36.2 ± 3.9% (\( n = 22 \); \( p < 0.001 \)) of control current (Figs. 4 and 5). A catalytically inactive Cys mutant of Nedd4 had no significant effect on \( \text{Na}^+ \) current amplitudes mediated by \( \text{Na} \),1,2 (100.9 ± 4.5%; \( n = 20 \)), \( \text{Na} \),1,7 (100.4 ± 4.3%; \( n = 20 \)), or \( \text{Na} \),1,8 (102.3 ± 9.9%; \( n = 15 \); Fig. 4).

In contrast, Nedd4-2 effectively abolished the \( \text{Na}^+ \) currents mediated by these three \( \text{Na} \) channel \( \alpha \)-subunits (Figs. 4 and 5). The \( \text{Na} \),1,2-mediated \( \text{Na}^+ \) currents were reduced to 25.3 ± 3.7% (\( n = 25 \); \( p < 0.001 \)), \( \text{Na} \),1,7 currents were reduced to 14.3 ± 3.1% (\( n = 20 \); \( p < 0.001 \)), and \( \text{Na} \),1,8 currents were reduced to 6.8 ± 8.8% (\( n = 30 \); \( p < 0.001 \)) of control currents. When oocytes were injected with 10-fold less Nedd4-2 cRNA, the inhibition of the \( \text{Na} \) currents was not as pronounced.
demonstrating that the effect was directly proportional to the amount of Nedd4-2 expressed (data not shown). Finally, we confirmed that the inhibition of the Na\(^+\) currents was due to Nedd4-2 ligase activity. Oocytes were injected with cRNA for the Cys mutant of Nedd4-2. In the presence of the ligase-deficient Nedd4-2 there was no effect on the peak Na\(^+\) current amplitudes (Fig. 4 and 5).

To further test whether the functional interactions between Na\(_v\) channels and Nedd4/Nedd4-2 is mediated via the PY motif in the carboxyl termini of these channels, we mutated this motif in Na\(_v\) 1.8 (PPSY) by replacing Tyr-1921 with an Ala (Y1921A). As shown in Fig. 6, the mutated channel was no longer inhibited by Nedd4-2 in Xenopus oocytes, indicating that PY motif is required for Nedd4-2-mediated regulation of Na\(_v\) 1.8 (n = 30).

**DISCUSSION**

Our data clearly demonstrate that all PY motif-containing Na\(_v\) channels can bind Nedd4 and Nedd4-2. We have further shown that all Na\(_v\) channels bind with the same WW domains (WW2 and WW3 of mouse Nedd4 and WW3 and WW4 of Nedd4-2) just as for ENaC subunits (10, 13). This is a striking similarity given that Nav channels and ENaC constitute two different classes of Na\(^+\) channels. We also found that subtle differences in WW domain preference occur between WW2 and WW3 of Nedd4 and WW3 and WW4 of Nedd4-2 for the tested Na\(_v\) channels. This may indicate a secondary role for the weaker interacting WW domain in each case, perhaps in binding to adaptor molecules.

We have also shown that the two Na\(_v\) channel \(\alpha\)-subunits tested can be ubiquitinated by Nedd4-2 and that the activities of all three Na\(_v\) channels tested are inhibited by Nedd4-2. Using a PY motif mutant of Na\(_v\) 1.8 we have further established that a functional interaction between the Na\(_v\) and the ubiquitin protein ligase is necessary for the inhibition of channel activity by the Nedd4-2 protein.

Interestingly, although all Na\(_v\) channels can bind WW domains from both Nedd4 and Nedd4-2, Nedd4 does not appear to ubiquitinate Na\(_v\) 1.2 and Na\(_v\) 1.7, the two Na\(_v\) channels tested in ubiquitination assays. On the other hand, in Xenopus oocytes the ectopic expression of Nedd4 can inhibit Na\(_v\) 1.2 and Na\(_v\) 1.7, although slightly less effectively than does Nedd4-2. These data suggest that Nedd4-2 and Nedd4 may differentially regulate various Na\(_v\) channels in vivo. It is possible that, whereas Nedd4-2 directly ubiquitinates and regulates Na\(_v\) channels, Nedd4 is involved in the ubiquitination of other proteins that regulate channel endocytosis and/or trafficking. Given that seven of the nine Na\(_v\) channels contain PY motifs in their carboxyl termini, we therefore propose that Na\(_v\) channels containing a PY motif are regulated by Nedd4/Nedd4-2-mediated ubiquitination.

 Trafficking and recycling are known to play important roles in the short term regulation of many ion channels; however, little is known regarding the regulation of Na\(_v\) channel activity by membrane insertion and retrieval. The main evidence for short term down-regulation of Na\(_v\) channels comes from studies in fetal rat brains, where activation of the channels by agonists such as scorpion \(\alpha\)-toxin or veratridine induces the internalization of the Na\(_v\) channels (18, 19). This channel down-regulation was mimicked simply by elevating intracellular Na\(^+\), demonstrating that the agonists induced a negative feedback effect due to elevated intracellular Na\(^+\) as a result of Na\(_v\) channel activation (18, 19). The mechanism underlying this effect remains unknown but appears to involve endocytic/lysosomal compartments (20) that may be mediated by Nedd4/Nedd4-2-dependent ubiquitination of Na\(_v\) channels.
REFERENCES

Regulation of Neuronal Voltage-gated Sodium Channels by the Ubiquitin-Protein Ligases Nedd4 and Nedd4-2
Andrew B. Fotia, Jenny Ekberg, David J. Adams, David I. Cook, Philip Poronnik and Sharad Kumar

doi: 10.1074/jbc.M402820200 originally published online April 27, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M402820200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 19 references, 8 of which can be accessed free at
http://www.jbc.org/content/279/28/28930.full.html#ref-list-1