Cross-kingdom auxiliary subunit modulation of a voltage-gated Sodium channel
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ABSTRACT

Voltage-gated, sodium ion-selective channels (Na\textsubscript{V}) generate electrical signals contributing to the upstroke of the action potential in animals. Na\textsubscript{V}S are also found in bacteria and are members of a larger family of tetrameric voltage-gated channels that includes Ca\textsubscript{V}S, K\textsubscript{V}S, and Na\textsubscript{V}S. Prokaryotic Na\textsubscript{V}S likely emerged from a homotetrameric Ca\textsuperscript{2+}-selective voltage-gated prognerator, and later developed Na\textsuperscript{+} selectivity independently. The Na\textsubscript{V} signaling complex in eukaryotes contains auxiliary proteins, termed beta (\(\beta\)) subunits, which are potent modulators of the expression profiles and voltage-gated properties of the Na\textsubscript{V} pore, but it is unknown whether they can functionally interact with prokaryotic Na\textsubscript{V} channels. Herein, we report that the eukaryotic Na\textsubscript{V}\(\beta\)-1-subunit isoform interacts with and enhances the surface expression as well as the voltage-dependent gating properties of the bacterial Na\textsubscript{V}, NaChBac in Xenopus oocytes. A phylogenetic analysis of the \(\beta\)-subunit gene family proteins confirms that these proteins appeared roughly 420 million years ago and that they have no clear homologues in bacterial phyla. However, a comparison between eukaryotic and bacterial Na\textsubscript{V} structures highlighted the presence of a conserved fold, which could support interactions with the \(\beta\)-subunit. Our the electrophysiological, biochemical, structural and bioinformatics results suggests that the prerequisites for \(\beta\)-subunit regulation are an evolutionarily stable and intrinsic property of some voltage-gated channels.

INTRODUCTION

Voltage-gated sodium channels (Na\textsubscript{V}) drive the upstroke of action potentials and are composed of a pore forming \(\alpha\)- and auxiliary \(\beta\)- subunits. The \(\alpha\)-subunits are large (~250 kDa) complexes that consist of 24-transmembrane helices that form four homologous (but not identical) domains, termed D\textsubscript{1}-D\textsubscript{4}. These heterotetrameric channels are sensitive to changes in transmembrane potential (voltage) and support the complex electrical signaling paradigms that underlie behavior, sensation, contraction and mobility (1-3). To date, ten sodium ion selective \(\alpha\)-subunits have been classified in humans. The four domain primary structure of eukaryotic voltage-gated calcium selective channels (Ca\textsubscript{v}) and Na\textsubscript{V}S is the result of two rounds of gene duplication which occurred in protists in a presumptive Ca\textsubscript{v} (4). Na\textsubscript{V}S subsequently evolved from Ca\textsubscript{v} in apoikozoa, a phylum comprising animals and the closely related choanoflagellates (5). In tandem, the emergence of the sodium selectivity and fast (sub-millisecond) voltage-dependent gating served as functional prerequisites for complex action potential firing and neuronal complexity (6). Further, five isoforms of sodium channel \(\beta\)-subunits have been identified, namely, \(\beta\)1, \(\beta\)2, \(\beta\)3, \(\beta\)4 and splice variant \(\beta\)1b (7-12). These 30-36 kDa proteins are comprised of an extracellular amino-terminus immunoglobulin-like domain, followed by an extracellular juxtamembrane region, a single transmembrane segment and an intracellular carboxyl-terminus; except for the splice variant \(\beta\)1b which has a non-conserved carboxyl-terminus and lacks a transmembrane segment (9). Related Na\textsubscript{V}\(\beta\)-subunits genes are expressed in cardiovascular and nervous tissues where they are part of the V-set immunoglobulin superfamily of cell adhesion molecules that exert non-conduction related functions such as facilitating cell adhesion and cell migration (7,12-14). Na\textsubscript{V}\(\beta\)-subunits recruit \(\alpha\)-subunits to the surface membrane in conjunction with the extracellular matrix and adhesion molecules ankyrin and contactin (13,15-20). Such interactions mediate sodium channel and K\textsubscript{V}7 clustering to the axon initial segment and nodes of Ranvier that mediate the saltatory conduction in neurons (21-24). Na\textsubscript{V} modulation is also seen in a wide range of \(\alpha\) and Na\textsubscript{V}\(\beta\)-subunit combinations, where a prominent effect of the \(\beta\)-subunit is enhancement of current density by promoting trafficking of the \(\alpha\)-subunit to the plasma membrane and altering \(\alpha\)-subunit voltage-dependent gating (17,25-36). The extracellular domains of \(\beta\)-subunits interact with the pore-forming subunit via covalent (\(\beta\)2/4) and non-covalent (\(\beta\)1/3)-interactions (37-43). Modulation of channel voltage-dependent gating is achieved through the effects of glycosylation which functions through a charge shielding mechanism (44-46).

Inherited mutations in Na\textsubscript{V}\(\beta\)-subunit genes can result in a variety of human disorders, including severe epilepsy (47-50), cardiac
arrhythmia (51,52), and are associated with cancer (53) and neuropsychiatric disorders (54,55). The underlying stoichiometry, putative interacting surfaces and mechanisms of β-subunit modulation of Na$_V$S remains poorly understood. Recently, Na$_V$β-subunits have been shown to play a role in neuronal excitability through novel interactions with K$_V$s, suggesting Na$_V$β-subunits may influence the function of channel-types outside of the eukaryotic Na$_V$ gene family (56-58).

Related voltage-gated sodium selective channels have been identified in prokaryotes where they are thought to play diverse roles in pH regulation, flagella movement and oxidative phosphorylation (59-62). Among these bacterial sodium channels, NaChBac was first isolated from an alkaliphilic bacterium Bacillus Halodurans and has been shown to be sodium selective and gated by voltage (63). Structural studies of bacterial Na$_V$S (bNa$_V$s) indicate they are surprisingly similar to eukaryotic voltage-gated cation channels, with a ‘domain-swapped’ architecture of four voltage-sensing domains which surround a central, gated pore (64). Consistent with this observation, bNa$_V$s are phylogenetically related to eukaryotic Na$_V$S (eNa$_V$s) and Ca$_V$, but represent a distinct evolutionary lineage. Specifically, bNa$_V$s are the result of an early differentiation from bacterial K$_V$s which likely occurred before the emergence of eukaryotes, while eNa$_V$s appeared more recently. Notably, bNa$_V$s are characterized by a homotetrameric quaternary structure, in contrast to the pseudotetrameric single polypeptide eNa$_V$.

The functional and structural relationships between prokaryotic and eukaryotic channel types are not fully known. For instance, while eNa$_V$s and bNa$_V$s are both sodium selective, the mechanisms are markedly different (65,66). On the other hand, bNa$_V$s show a pharmacological profile similar to that of the eNa$_V$ family, an observation that suggests that the channels of these two phyla might share structural similarities that support mechanisms of activation and inactivation (67-69). Despite potential differences, bNa$_V$s have been the subject of extensive functional and structural studies that have provided key insights into mechanisms of cation recognition, drug binding, voltage-sensing and channel gating (67,70-79).

Here, we report that NaChBac is modulated by the mammalian voltage-gated sodium channel auxiliary β1-subunit in Xenopus laevis oocytes. β-subunit modulation of NaChBac was isofrom specific, β1 strongly enhanced current amplitudes by affecting cell surface expression and the voltage dependence of NaChBac gating, while β2 marginally decreased cell surface expression of the channel resulting in modest sodium current amplitudes. Further, in HEK293T cells, β1 and β3 but not β2, co-purified a co-expressed NaChBac indicating that the functional modulation of NaChBac is the product of the formation of a novel protein complex comprised of a bacterial sodium selective pore and a eukaryotic β-subunit. The reciprocal purification of NaChBaC and co-purification of β1 and β3, but not β2 indicates the specificity of this interaction. An evolutionary analysis of the β-subunit V-set immunoglobulin family pinpoints their emergence in osteichthytes, roughly 420 MYA, and failed to identify related protein families in bacteria. Modelling analysis of the interaction regions between β1 and NaChBac based on the cryo-EM interaction between Electric Eel β1 and Na$_V$.1.4 indicate a conserved interaction of polar residues in the protein-protein interface between the two α-subunits. In total, the data suggests that the gene family that comprises the sodium channel auxiliary β-subunits may act as broad regulators of electrical signaling through the interaction and modulation of multiple channel types. This functional plurality may arise from the strict evolutionary conservation of specific structural elements found in the voltage-gated ion channels.

RESULTS

Eukaryotic Na$_V$β-subunits are known to modulate eukaryotic Na$_V$ channels, (3,80), and recently this functionality has been extended to the potassium channel isoforms K$_V$1, K$_V$.4.2/4.3 and K$_V$.7 (56-58). To test the possibility that eukaryotic β-subunits may also modulate bNa$_V$s, cRNA encoding NaChBac was co-injected with eukaryotic Na$_V$β1 and β2 in Xenopus oocytes, an expression system that lacks endogenous β-subunit expression, unlike a majority of mammalian cell lines (3,81-85). Co-expression of NaChBac with β1 in Xenopus oocytes significantly increased sodium current amplitudes over NaChBac alone and this trend was sustained over a 50-hour time course (Fig. 1A,
B, C). NaChBac injected alone resulted in currents roughly 36 hours post injection while NaChBac/β1-mediated currents were significantly above background 12 hours after injection (Fig. 1A). Saturation of the functional expression was not observed, but NaChBac/β1-mediated currents exceeded the level of reliable voltage clamp after 48 hours (Fig 1A). In contrast, the β2 effect on NaChBac-mediated currents was marginal (Fig. 1A, B, C) and small sodium currents were measurable only 36 hours post injection and did not increase over longer incubation time (Fig. 1A).

Evidence of a possible β-subunit interaction with NaChBac, either direct or via a complex, was obtained by affinity purification. β1, β2 and β3 were isolated from protein lysates by a C-terminal 6x His-tag using a Ni-NTA column. HEK293T cells transfected with NaChBac/β1 and NaChBac/β3 showed association via co-pulldown of NaChBac. This was observed by immunoblotting for a NaChBac N-terminal HA probe that was detected in the elution fraction upon pulldown of the β-subunit, which were identified via the V5 C-terminal tag on the β-subunits. Ni-NTA pulldown of NaChBac/β2 showed pulldown of the β-subunit, observed via a C-terminal HA tag, however NaChBac was not detected in the elution for NaChBac/β2 or NaChBac only (Fig 2A). This result was replicated with NaChBac pulldown using Strep-Tactin Sepharose resin against a N-terminally Twin-Strep tagged NaChBac, whereby both β1 and β3 co-purified with NaChBac however β2 was not observed in the NaChBac purification elution (Fig 2B). This reciprocal purification suggests either a direct interaction or the composition of a complex that is tightly associated with both NaChBac and the β1/3-subunits. However, the presence of an endogenous β1-subunit expressed in HEK293T cells precluded electrophysiological studies of NaChBac and co-expressed NaV β-subunits. Further, cell lines which lack β1, such as CHL or COS cells, may contain β1b or β3, the latter being a post-transcriptionally regulated soluble β-subunit that is capable of regulating NaV,δ (86).

In order to determine the possible mechanism of current enhancement by the β1-subunit we assayed the plasma membrane appearance of NaChBac by biotinylation of cell surface proteins in the presence and absence of β1 or β2 in Xenopus oocytes. NaChBac surface expression was detectable biochemically at the plasma membrane after 24 hours post-injection and the total (surface + cytoplasmic) expression was comparable in all three conditions (Fig. 1D). However, the biotinylated (plasma membrane) fraction indicates co-expression of β1 promoted the surface expression of NaChBac while β2 co-expression resulted in a reduction of surface located channels. Densitometric analysis of the biotinylation data reveals that β1 increases NaChBac surface expression by approximately 450% while the β2-subunit reduced expression by approximately 30% (Fig. 1D).

Given that the data demonstrated that β1 was able to form a stable protein complex with NaChBac and increase the cell surface channel density, we next assessed possible effects on voltage-gated properties of NaChBac expressed in Xenopus oocytes. First, a variable length depolarizing pulse was used to minimize the effect of channel inactivation on the tail-current. Example traces (Fig. 3A) for NaChBac-, NaChBac/β1- and NaChBac/β2-mediated currents used for analysis of the steady state activation p_{open} with the resulting tail-current GV curves (Fig. 3B) are depicted. Co-expression of β1, but not β2, left-shifted and steepened the GV relationship, compared to NaChBac alone (table 1). Second, and similarly, β1 left-shifted the voltage dependence of NaChBac steady-state inactivation while β2 did not (Fig. 3C). Lastly, β1 accelerated the voltage-dependent activation and inactivation kinetics (Fig 3D/E).

These results suggest a vestigial conservation between prokaryotes and euukaryotes α/β-subunit structural interaction interface. This hypothesis raises several fundamental questions: do bacterial counterparts of β-subunits exist? If so, when did β1/3 and β2 begin to functionally diverge? To answer these questions, we performed a bioinformatics analysis aimed at tracing the evolutionary history of these proteins. Whereby, we scour the comprehensive Uniprot database to identify genes homologous to human β1-subunits in other genomes.

This genome-wide scan identified an extensive set of diverse sequences including, as expected, the genes encoding β2, β3 and β4 isoforms in different organisms and provided some
insights into their evolutionary history. First and foremost, all identified β-subunit genes are from eukaryotes and, in particular, metazoa. Second, the branch of the dendrogram containing the β-subunits highlights an interesting hierarchy of sequence similarities: β1 and β3 are closer to each other than to the pair formed by β2 and β4. Thus, it appears that the functional divergence between β1 and β2 predates the gene duplication event that gave rise to β3 and β4. We therefore decided to further investigate the close evolutionary relationship between β1 and β3 by inferring their phylogenetic tree (Fig. 4). We found that β1 and β3 have a relatively recent evolutionary history: these genes are found in euteleostomi (bony vertebrates) but not in chondrichthyans (cartilaginous fishes). This observation indicates that the last common ancestor between β1 and β3 occurred around 420 MYA, and the absence of β2 in chondrichthyans further confirms that β-subunits were not present before the emergence of euteleostomi and therefore unlikely be present in prokaryotes. Further, this possibility extends to the greater family of V-set proteins (http://pfam.xfam.org/family/PF07686.15#tabview=tab7) containing the immunoglobulin domain, that is not identified in prokaryotes, that is sufficient for β-subunit modulatory effects (39,40,87).

Overall, the evolutionary history of β-subunits and, in particular, the lack of a recognizable prokaryotic homologue suggests that the major structural elements of NaVαs involved in the α/β interactions are conserved across kingdoms. Indeed, a conserved structural platform for docking β-subunits would explain the interaction of a human auxiliary subunit with a voltage-gated bacterial channel. To examine this possibility, we analyzed the recently determined structure of the complex between β1 and NaV1.4 from electric eel obtained via cryo-electron microscopy (41). This structural information revealed that, besides the interactions between the immunoglobulin domain of β1 and the extracellular loops of NaV1.4, a large set of residue-residue contacts are present between the transmembrane regions of the two proteins. In particular, the transmembrane helix of β1 shares an extended protein-protein interface with the membrane boundary S0 and transmembrane S2 of NaV1.4 DIII. Importantly, this “bidentate” interaction, involving simultaneous contact with the voltage sensor domain and the extracellular part of the pore, dictates a precise arrangement between these two parts of the channel. Utilizing this structure as a template for the β1-α binding domain, we set out to examine these interaction elements in NaChBac. However, since an experimentally derived structural model of NaChBac is not available, we considered the recently determined structure of the full-length model of NaVMs (88). This channel has been extensively electrophysiology characterized and, as NaChBac, has been shown to possess a pharmacological profile very similar to that of eNaVs (68). We performed a structural superposition between NaV1.4 and NaVMs using MISTRAL (89), an algorithm that finds the optimal rotations and translation to minimize the root mean square deviation (RMSD) between corresponding Cα atoms in two proteins. In this case, MISTRAL identifies the two transmembrane regions as structural homologues and returns a remarkably small RMSD value (2.3 Å). This is indicated by the closely superimposed pore domains (Fig. 5A-B), divergence is mainly from the voltage sensors, with the exception of DIII. Thus, it appears that only DIII, the domain involved in the α/β interaction, has conserved the “ancestral” arrangement with respect to the pore domain.

Having ascertained that the structure of a βNaV can provide an interaction interface for the β-subunits similar to that of eNaVs, we focused our attention on the specific networks of residue-residue interactions between the two proteins. To this end we considered a homology model of NaChBac based on the structural template of NaVAb and subject to multi μs molecular dynamics simulations (90,91). Despite being a theoretical prediction, this structural model has been repeatedly experimentally validated (92,93). The superposition of NaChBac and NaV1.4 reveals a structural match as good as the one observed for NaVMs. Moreover, a peculiar pattern emerges in the sidechain chemical identity at the protein-protein interface: there are two clusters of polar residues, one in the middle of the membrane and the other more displaced toward the extracellular compartment that are present in both NaChBac and NaV1.4 that engage in directional interaction
with hydrophilic residues on β1 (Fig. 5C). We therefore examined the evolutionary pattern of the polar transmembrane helices as a potential basis for a NaChBac preferential interaction with β1/3 over β2/4.

To address these issues, we analyzed comprehensive multiple sequence alignments of eNaV_S and bNaV_S containing thousands of genes from all sequenced organisms (4). Surprisingly, the residues of S2 involved in the interaction with β1 are the least conserved, not only in bNaV_S, but also in eNaV_S (Fig. 6A). The absence of a statistical signal of sequence conservation suggests that the α/β interaction might not be absent in many organisms. An “intermittent” presence of a protein-protein interaction, whereby mutation of one or two residues switches “on” or “off” an interaction in different organisms, is not uncommon in evolution and might be at work also in the case of β-subunits (94). However, despite the poor conservation of the contacting residues, a separate analysis of the sequences encoding for DIII reveals a prevalence of hydrophilic residues at the positions where the polar interactions take place (Fig. 6B). This conservation is, to some extent, present also in bacterial channels but not, for instance, in eukaryotic DIII.

DISCUSSION

Eukaryotic NaV_β-subunits are multifunctional transmembrane proteins that can have numerous modulatory effects on the expression and function of the pore-forming NaV_α-subunit. In addition to the well-known effects on NaV biophysical characteristics by β-subunits, they play important roles in cellular maintenance as adhesion molecules that interact with cytoskeletal ankyrin and contactin proteins, interactions that can also enhance the functional expression of sodium channels at the cell surface (21-24). In the present study, the data shows that the eukaryotic NaV_β1-subunit significantly increases the peak current and functional properties of the bacterial channel NaChBac in Xenopus laevis oocytes. By contrast, NaV_β2 modestly influenced the number of functional NaChBac channels at the plasma membrane, with no significant effects on channel function. The results surprisingly mirror β-subunits isoform specific surface enhancement of cardiac NaVs by the β1-subunit, but not by β2 (95).

β1 co-expression induced hyperpolarizing shifts in the voltage dependence of both activation and inactivation of NaChBac. Additionally, this functional regulation was supported via a biochemical pulldown assay in mammalian HEK293T cells whereby heterologously expressed NaChBac co-affinity purified with NaV_β1 and NaV_β3 but not with NaV_β2 the reciprocal purification was also observed whereby NaV_β1 and NaV_β3 co-purified with NaChBac while NaV_β2 did not. Further, co-expression of NaVs with the β1-subunit has been shown to increase the peak of brain sodium current and to shift the voltage dependence of inactivation (38). It is not known for eNaV_S if the same underlying interactions and mechanisms are responsible for β-subunit-induced surface membrane enhancement and tuning of voltage-gating. Recent cryo-EM evidence shows a direct interaction of β1 extracellular domain with the NaV voltage-sensing domain, the β1 interacting domain is highly conserved with the β3-subunit and we theorize this to be the interface of not only the β1 but also the β3-subunit interaction with NaChBac observed in our co-purification conditions (41). NaChBac lacks any cysteine residues to form a disulfide bond with the β2/4 extracellular domain that is necessary in β-subunit covalent interaction with NaVs and localization to Nodes of Ranvier (96). NaV_S have a presumed 1:1 ratio with the β-subunit yet this is believed to interact with a distinct voltage-sensing domain while NaChBac like KVs have multiple identical voltage-sensing domains, making it unclear if the stoichiometry of the NaChBac/β-subunit complex is conserved (97). The amenable nature of bNaV structural studies makes it conceivable that a structure of a bNaV/β-subunit complex is feasible and such studies could shed light on the general mechanism(s) of β-subunit regulation.

The current density arising from the inward flux of sodium ions is related to the total number channels in the surface membrane, the amount of time that they spend in the open conformation (open probability), and the amount of sodium ions that flow through the open channel (unitary conductance). However, up-regulation of mammalian sodium current by β1 co-expression in both native settings and in heterologous expression systems can be simply explained by enhanced
surface expression (25,51), similar to the effect here by β1 on NaChBac. Yet, for eNaV's or bNaV's the data cannot distinguish between enhanced forward trafficking, enhanced surface stability or diminished endocytic and/or lysosomal retrieval. Regardless, the enhanced surface expression of NaChBac by co-expression with β1 may have practical applications in the study of other bNaV's that have shown poor expression profiles in heterologous systems.

The extent to which eNaV's can be modulated by β-subunits can depend strongly on the expression system. One possible cause of this functional variability may be the wide-spread endogenous expression of NaVβ-subunits in mammalian cell lines (3,15,98,99). Unlike HEK293T cells, Xenopus laevis oocytes do not have a known β-subunit thus motivating their use for the present study (98,99). Interestingly, NaChBac demonstrates robust expression in HEK293T cells in our hands but these currents were not obviously modulated by either β1 or β2 expression, possibly owing to a competing interaction with the expressed channel such protein being expressed in HEK293T and not oocytes may be an endogenous β-subunit (not shown). Thus it is possible that the previous reports of expressed NaV's (100) or NaChBac currents in HEK293T or tSA 201 cells (65,101,102), may have been, in fact, channels associated with endogenous β-subunits.

An evolutionary analysis of the β-subunits family reveals that these auxiliary subunits are part of a large family comprising several myelin associated proteins. Moreover, the phylogenetic tree of these genes reveals that β1/β3 and β2/β4 form two distinct groups. Strikingly, a recent cryo-EM structure of the complex between NaV1.4 and β1 shows that the part of the channel involved in the protein-protein interaction is structurally superimposable to the corresponding region in NaChBac, even though the individual amino acids involved in the α/β interaction are not conserved across evolution.

In total, the electrophysiological, biochemical, structural and bioinformatics data suggests that the structural prerequisites for β-subunit regulation are an evolutionarily stable, intrinsic property of some voltage-gated channels, including bNaVs, that were present before the emergence of the auxiliary proteins.

**EXPERIMENTAL PROCEDURES**

**Expression of NaChBac and β-subunits in Xenopus oocytes** – cRNA of NaChBac, rat β1, β2 were synthesized in vitro (Ambion, mmessagemm machine, T7) and co-injected into Xenopus oocytes (50 ng of total cRNA/oocyte) with 1:1 ratio of NaChBac and DEPC treated water or with beta subunits. The injected oocytes were kept at 18°C in oocyte ringer solution 3 (OR3) containing 50% Leibovitz’s L-15 Medium, 10 mM HEPES, 0.5% Glutamine and 0.5% Gentamycin, pH 7.6. Electrophysiological measurements were performed at various time points post injection. Cell surface biotinylation was performed at 24 h post injection.

**Two Electrode Voltage Clamp** – Voltage-clamped sodium currents were recorded with two microelectrodes using an OC-725C voltage clamp (Warner, Hamden, CT) in a standard Ringers solution (in mM): 116 NaCl, 2 KCl, 1 MgCl2, 0.5 CaCl2, 5 HEPES (pH 7.4). Current recordings were performed at 20-22°C. Glass microelectrodes had resistances of 0.1 - 1 MΩ and were backfilled with 3 M KCl. The holding potential was -120 mV in all cases to minimize current rundown. Conductance-voltage (GV) relationships were derived by plotting the isochronal tail current amplitudes (the current amplitude measured after stepping from the test potential back to a holding potential of -120 mV) as a function of the depolarizing pulse potential. Depolarizing pulses were delivered as envelope protocols to minimize the contribution of inactivation in GV analysis. Displayed current traces show the full active voltage range of GV's. All data are mean ± SEM.

**Biochemical Detection of NaChBac**

**Surface Expression** – Biotinylation was performed using 63 oocytes per experimental condition. All biotinylation procedures were performed as previously described (103). Briefly, anti-HA high affinity antibody (Roche (USA) 11867423001), directed against an HA peptide on the N-terminus of NaChBac was used because C-terminal HA-tag on NaChBac increased the current amplitude and abolished modulatory effects of β1 and β2. Mouse monoclonal antibody directed against α-tubulin (T6199) was purchased from Sigma. Both anti-rat...
(SAB3700526) and anti-mouse (SAB3700993) horseradish peroxidase conjugated secondary antibodies were obtained from Sigma. HA-tagged NaChBac was detected by incubating the membrane with 1:1,000 diluted anti-HA at 4°C overnight and 1:10,000 of anti-rat secondary antibody incubation for 1 hour at room temperature. The nitrocellulose membrane was stripped with mild stripping buffer as described Abcam-stripping for reprobing protocol. For tubulin detection in each sample, membrane was incubated with anti-tubulin (1:1,000) for 3 hours at room temperature, followed by 1 hour room temperature incubation with anti-mouse (1:5,000). ImageJ software (NIH) was used to quantify band densities.

Expression and His-tag or Strep-Tactin Sepharose purification of NaChBac and β-subunits in HEK293T cells – HEK293T 10 cm plates were transfected with 2.5 μg of NaChBac (N-terminal HA or Twin-Strep Tag) and/or rat β1 (C-terminal V5 and 6xHis), β2 (C-terminal HA and 6xHis), β3 (C-terminal V5 and 6xHis) plasmid using 15 μl of LipoD293 or Polyjet (SigmaGen, SL100668, SL100688) 24 hours post trypsinization at confluences of ~60-70%. Two days post transfection cells were harvested and washed with PBS containing 1 mM Benzanidine and PMSF. Pelleted cells were lysed for 10 mins in lysis buffer: 150 mM NaCl, 100 mM Tris-HCl pH 8.0, 1% Triton X-100 and a mini cOmplete protease inhibitor tablet (Roche, 118361530). Removal of cellular debris was accomplished by a 20 minute 20,000 xg spin at 4°C. His Tag purification: Lysate was diluted in half with PBS and imidazole to a final 10 mM imidazole concentration before being loaded onto a 250 ul PerfectPRo Ni-NTA agarose column (5Prime, 2900510). The column was washed 4x with 1ml of PBS + 0.1% Triton-X 100 and 20 mM imidazole and eluted with 1 ml of PBS + 0.1% Triton X-100 with 500 mM imidazole and concentrated 50-fold with an Amicon Ultra 10 kDa concentrator (UFC501024). Fractions were loaded onto a 9% polyacrylamide gel (1% total lysate, 1% pulldown fraction or 3% elution for β-blots or 12% elution for NaChBac blots) and run out at 50V for 30 min, 150V for 1 hour. Protein was transferred to PVDF (GVS NA, 1212637) at 100V for an hour. Strep-Tactin Sepharose Purification: Lysate was loaded onto a 100 μL Strep-Tactin Sepharose column (IBA, 2-1201-002) and flow through 3x. The column was washed with 23 mL of wash buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 0.1% triton-X 100) and eluted using wash buffer containing 10 mM desthiobiotin (Sigma, D1411) with 3x 300 μL elutions, of which the second eluent contained ~80% of protein. Lysate and elution fraction were loaded onto a 9% polyacrylamide gel (1% total lysate, 6.7% of second elution) and run out at 50V for 30 min and 150V for 1 hour. Protein was transferred to nitrocellulose (GE Healthcare, 10600002) at 100V for an hour. Blots were block for 1 hour with either 3% cold water fish gelatin (Sigma, G7041) or 5% milk in TBST. Primary antibody 1:1,000 anti-HA (ThermoFisher, 2618) or 1:20,000 anti-V5 (ThermoFisher, R960-25) were incubated overnight at 4°C in blocking buffer. Primary was washed off with 3x 10 min TBST (0.1% Tween-20) washes before secondary 1:10,000 anti-Mouse IgG1 specific (ThermoFisher, A10551) for HA and 1:1,000 anti-Mouse IgG (Jackson Immuno Labs, 115-035-003) for 1 hour before washed off with 3x 10 min TBST washes. Strep-Tactin HRP (Bio-Rad, 1610381) was used according to manufacturer’s stipulations at 1:20,000. Blots were incubated for 5 minutes in Clarity ECL solution (Bio-Rad, 1705060) before being imaged. Blots shown are representative of three replicates.

Bioinformatics analysis of β-subunits - A multiple sequence alignment was obtained using first HMMER (Finn et al., 2011) to scan the Uniprot database (RP35 proteome), using phmmer tool to collect homologous sequences of human β1 and β2 sequences. These were then accurately aligned with MAFFT and redundant sequences with pairwise identity higher than 95% removed, in order to obtain a first general alignment (104). From the derived phylogenetic tree the branches containing Swissprot-annotated β1 and β3 sequences were extracted and the relative sequence realigned with MAFFT to obtain a more refined tree. All the trees were generated using FastTree (Price et al., 2010); the statistical significance of each branch was estimated using the bootstrap approach (a posterior probability higher than 0.7 is considered significant).
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The abbreviations used are: eNa\textsubscript{V}, eukaryotic voltage-gated sodium channel; bNa\textsubscript{V}, bacterial voltage-gated sodium channel; Voltage gated sodium channels, Na\textsubscript{V}; voltage-gated sodium channels, Na\textsubscript{V}s; GV, conductance-voltage relationship; SSI, steady-state inactivation; \(\beta\), voltage-gated sodium channel beta subunits.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest with the contents of this article.

AUTHOR CONTRIBUTIONS
SM, SL and LL performed experiments and analyzed data; DG and VC performed evolutionary relationships; SM, JDL and CAA designed research; all authors contributed to the paper.
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| Vm (mV) | NaChBac (7) | NaChBac+β1 (7) | NaChBac+β2 (6) | NaChBac (7) | NaChBac+β1 (7) | NaChBac+β2 (6) |
|--------|-------------|----------------|---------------|-------------|----------------|---------------|
| -30    | 25.2±1.1    | 12.4±0.8*      | 19.9±0.8*     | -40         | 906.6±56.9     | 579.8±21.7*   | 780.4±50.5   |
| -20    | 16.0±0.8    | 7.1±0.4*       | 12.2±0.3*     | -30         | 654.5±30.4     | 528.1±16.6*   | 583.7±14.7   |
| -10    | 9.6±0.5     | 4.7±0.3*       | 7.6±0.1*      | -20         | 571.1±17.5     | 496.6±16.2*   | 530.7±5.4    |
| 0      | 6.5±0.4     | 3.3±0.3*       | 5.0±0.2*      | -10         | 539.4±13.5     | 476.8±16.1*   | 511.5±4.3    |
| 10     | 4.7±0.3     | 2.6±0.3*       | 3.6±0.2       | 0           | 524.8±11.7     | 467.5±16.5*   | 507.5±7.3    |
| 20     | 3.7±0.2     | 2.2±0.3*       | 2.9±0.2       | 10          | 527.4±11.9     | 467.9±18.8*   | 518.4±17.4   |

Table 1. Effects of eNaβ-subunits on NaChBac activation and inactivation Conductance-voltage relationship and steady-state inactivation protocol was fit with a Boltzmann function. Mean values of V_{1/2} and the slope (dx) of the fit are depicted in mV with number of samples used to calculate noted in brackets. Statistical significance was set at P <0.05 and marked with asterisks.
Table 2. eNa\(\beta\)-subunits affect the gating kinetics of NaChBac. Time constants were obtained from single exponential fits of activation and inactivation kinetics at various voltages. Bracketed values represent sample replicates used to calculate the mean and SEM. Statistical significance was set at P < 0.05 and marked with asterisks.

|        | NaChBac | NaChBac + \(\beta\)1 | NaChBac + \(\beta\)2 |
|--------|---------|----------------------|----------------------|
| GV     | V\(_{1/2}\) | -30.7 ± 1.5          | -44.6 ± 0.8*         | -27.5 ± 3.8          |
|        | dx      | 11.8 ± 0.7           | 7.6 ± 0.3*           | 10.5 ± 0.7*          |
| SSI    | V\(_{1/2}\) | -51.1 ± 0.7          | -57.8 ± 0.3*         | -52.0 ± 0.4          |
|        | dx      | 5.1 ± 0.2            | 5.5 ± 0.2*           | 4.7 ± 0.4*           |
FIGURE 1. Isoform specific modulation of the bNaV NaChBac by eNaVβ-subunits (A) top, representative currents for NaChBac expressed alone (filled circles) or in the presence of the β1-(open circles) or β2-(open squares) subunit 48 hours post-injection, scale bars represent 2 µA and 10 milliseconds. (A) bottom panel, β1 promotes an increase in current density. Co-injection of β1-subunit increases the current of NaChBac while β2 mildly reduces the current amplitude as compare to NaChBac.
alone. (B) Current-voltage relationship 42 hours post-injection. (C) Peak current (at -20mV) of NaChBac with β-subunits at indicated time-points. (D) Surface expressed NaChBac channels assessed by channel biotinylation at 24 hours post-injection. Left, total expression of NaChBac in the supernatant fraction is not altered by co-expression of the β1 or β2 subunit. Anti-tubulin serves as a loading control. Right panel indicates the surface expression of NaChBac is increased by β1 but not β2 (left to right), see Methods for experimental details. Absence of tubulin in the biotinylated fraction ensures biotinylation of surface proteins only.
A NaChBac Purification

![Diagram of NaChBac Purification]

**FIGURE 2.** NaChBac and β-subunit reciprocal purification. (A) Blots depict β1 and β3 co-purified with NaChBac Strep-Tactin purification. Shown are lysate and elution fractions from Strep-Tactin Sepharose pulldown of N-terminally Twin-Strep Tag NaChBac, left section, and expression and co-purification of β-subunits with the NaChBac pulldown, right 3x sections, from HEK293T cells. (B) Blots depict NaChBac co-purified with β1 and β3. Shown Ni-NTA pulldown fractions: flow through, washes and elution of His-tag containing β-subunits from HEK293T cells transfected with NaChBac alone or NaChBac + β1, + β2 or +β3. See Methods for details.
FIGURE 3. β-subunit alters biophysical properties of NaChBac (A) Representative current traces of NaChBac alone, + β1 or + β2, at 48 hour, 24 hour or 48 hours post injection, respectively. In each case the scale bar represents 50 ms and 1 µA. Envelope of tails protocol with diminishing depolarization duration was used to minimize the effect of channel inactivation on tail current conductance-voltage analysis, see Methods for details. (B) Normalized conductance-voltage (GV) relationships show that β1 (open circles) left-shifts and steepens the GV relationship, a representative envelope protocol is shown in
Inset with parameters from the Boltzmann fit tabulated as Table 1. (C) Steady-state inactivation (SSI) (protocol shown in the inset) shows β1 induces a statistically significant hyperpolarizing shift in the SSI relationship. SSI protocol is shown in the inset; 3 s prepulse (PP) at various potential is followed by 37.5 ms of test pulse (TP) at -20 mV. Oocytes are held at holding potential of -120 mV throughout the experiments. (D) Co-expression of β1-subunit accelerates activation kinetics of NaChBac. Inset: normalized activation curves of NaChBac only (filled circle), NaChBac co-expressing β1 (open circle), NaChBac with β2 (open square). Scale bar represents 20 ms. (E) β1-subunit speeds the inactivation kinetics of NaChBac. Inset: representative traces of WT NaChBac only, NaChBac with β1 and NaChBac with β2 (left to right) with single exponential fit. Scale bar denotes 500 ms.

**FIGURE 4.** Evolutionary history of β1 and β3. Phylogenetic analysis of the sequences segregating in the branch containing β1 and β3 of the general dendrogram (Fig. 4). All the major nodes appear to be well supported (see numbers). Note that β1 (SCN1B) and β3 (SCN3B) are present in the same set of vertebrates (sarcopterigii and actinopterigii) and that no genes from chondrichthyes (cartilaginous fishes) are present in the tree.
FIGURE 5. Structural determinants of the α/β interaction. Structural alignment between NaV1.4 and the bacterial channel NaVMs: top (A) and side (B) views. Each domain is rendered using a different color: D_I is shown in purple, D_II in cyan, D_III in blue and D_IV in light brown. NaVMs and the β1-subunit bound to NaV1.4 are shown in white and red, respectively. Note that, while the voltage sensor from D_III is perfectly superimposed, the same domain from D_I or D_II (purple and cyan, respectively) does not show the same degree of structural similarity. C) Structural alignment between S0-S2 of NaV1.4 D_III (blue) and NaChBac (white); the β1-subunit bound to NaV1.4 is shown in red. Sidechains engaged in residue-residue contacts across the protein-protein interface are shown as sticks colored according either the non-polar (white) or polar (green) character of their side chains.
FIGURE 6. Evolutionary conservation of eNaVs and bNaVs and of β-subunits. (A) The sequence logos show the conservation profile of the genes encoding for eukaryotic and prokaryotic NaVs in all sequenced organisms. Only the voltage sensor domain is shown. Light blue shading highlights the residues involved in the interaction with β1. Note how these positions are the least conserved within the voltage sensor domain. (B) Sequence logos of the S2 segment from DIII, prokaryotic channels and DII the cyan arrows highlight the residues involved in polar interactions with β1. (C) Sequence logos of the transmembrane region of the genes orthologous to β1 and β2. The light blue shading highlights the residues involved in polar interactions with S2 of the NaV channels.
