

Distinct domains of the sodium channel β 3-subunit modulate channel-gating kinetics and subcellular location

Esther J. YU*, Seong-Hoon KO†, Paul W. LENKOWSKI†, Alena PANCE*, Manoj K. PATEL† and Antony P. JACKSON*¹

*Department of Biochemistry, The University of Cambridge, Downing Site, Tennis Court Road, Cambridge CB2 1QW, U.K., and †Department of Anesthesiology, The University of Virginia Health System, Charlottesville, VA 22908, U.S.A.

Electrical excitability in neurons depends on the expression and activity of voltage-gated sodium channels in the neuronal plasma membrane. The ion-conducting α -subunit of the channel is associated with auxiliary β -subunits of which there are four known types. In the present study, we describe the first detailed structure/function analysis of the β 3-subunit. We correlate the effect of point mutations and deletions in β 3 with the functional properties of the sodium channel and its membrane-targeting behaviour. We show that the extracellular domain influences sodium channel gating properties, but is not required for the delivery of β 3 to the plasma membrane when expressed with the α -subunit. In contrast,

the intracellular domain is essential for correct subunit targeting. Our results reveal the crucial importance of the Cys²¹–Cys⁹⁶ disulphide bond in maintaining the functionally correct β 3 structure and establish a role for a second putative disulphide bond (Cys²–Cys²⁴) in modulating channel inactivation kinetics. Surprisingly, our results imply that the wild-type β 3 molecule can traverse the secretory pathway independently of the α -subunit.

Key words: auxiliary β -subunit, channel-gating kinetics, extracellular domain, intracellular targeting, site-directed mutagenesis, sodium channel.

INTRODUCTION

Voltage-gated sodium channels play a fundamental role in all electrically excitable cells and tissues. They respond to an increase in the membrane potential by transiently enhancing the sodium permeability of the plasma membrane and, in neuronal axons, they help to propagate the action potential [1,2]. Sodium channels are major targets for anticonvulsants and local anaesthetics [3] and mutations in sodium channel genes have been implicated in a variety of pathologies including epilepsy, cardiac arrhythmias such as long QT syndrome and Brugada syndrome, and some muscle myotonias [4–6].

The sodium channel contains a central 260 kDa α -subunit that forms the ion pore and dictates the channel specificity. There are at least ten distinct α -subunits that vary in their tissue distribution and kinetic properties [1]. Sodium channels also contain two functional classes of auxiliary subunits, of which β 1 and β 2 are the originally identified members [7,8]. A third auxiliary subunit, β 3, is structurally most similar to β 1, and a fourth subunit β 4 is homologous with β 2 [9,10]. The β 3- and β 1-subunits share only approx. 50% amino acid sequence identity and show clearly distinct expression patterns both within and between tissues and during embryonic and postnatal development [9,11]. This suggests that despite their overall sequence similarity, β 1 and β 3 play distinct roles. The β 3-subunit increases the fraction of channels operating in the fast-gating mode and thus enhances the activation and inactivation gating kinetics of the channel [9]. Under some conditions, the β 3-subunit increases the expression of the sodium channel at the plasma membrane. Thus it may help either in the assembly and trafficking of the channel to the surface or in channel retention at the plasma membrane [12]. However, unlike β 1 [13–15], there have so far been no published studies that correlate structure and function within the β 3-subunit. In the present study, we use site-directed mutagenesis to identify distinct

domains on the β 3-subunit that modify the electrophysiological properties of the sodium channel. In addition, we examine how mutations in β 3 affect the intracellular location of the subunit and the extent to which the mistargeting of the mutations correlates with their electrophysiological phenotypes.

MATERIALS AND METHODS

Generation of EGFP (enhanced green fluorescent protein) fusion constructs

PCR was used to amplify the open reading frame of β 3 from full-length cDNA. The 3'-primer was designed to exclude the TAA stop codon and to include an AgeI site. The PCR product was subcloned into the pGEM T Easy vector (Promega), which was then cut with SacII and AgeI to generate a SacII–AgeI fragment containing the β 3-subunit. The fragment was ligated in-frame into the SacII–AgeI site of the vector pEGFP-N1 (Clontech). The ATG start codon for EGFP was deleted using the QuikChange XL site-directed mutagenesis (Stratagene), to ensure that EGFP transcription did not occur separately from β 3. The final fusion construct encodes the β 3 signal sequence with the full protein sequence of β 3 (amino acids 1–191) [9] together with EGFP connected via the amino acid sequence EPVAT as a linker region (Figure 1). This construct was used as the template for all subsequent mutations. Δ ECD (ECD-deletion mutant, where ECD stands for extracellular domain) and Δ ICD (ICD-deletion mutant, where ICD stands for intracellular domain) were made by removing the relevant regions in the β 3-subunit using PCR. The Δ ECD mutation is a deletion of the extracellular region of β 3 (amino acids 1–135) with the putative signal sequence left intact. The Δ ICD mutation is a deletion of the intracellular region of β 3 (amino acids 157–191). The cysteine mutants C24A and C96A were made by site-directed mutagenesis using the Stratagene kit as

Abbreviations used: CHO, Chinese-hamster ovary; ECD, extracellular domain; Δ ECD, ECD-deletion mutant; ICD, intracellular domain; Δ ICD, ICD-deletion mutant; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; Ig, immunoglobulin; NGF, nerve growth factor; TMD, transmembrane domain.

¹ To whom correspondence should be addressed (email a.p.jackson@bioc.cam.ac.uk).

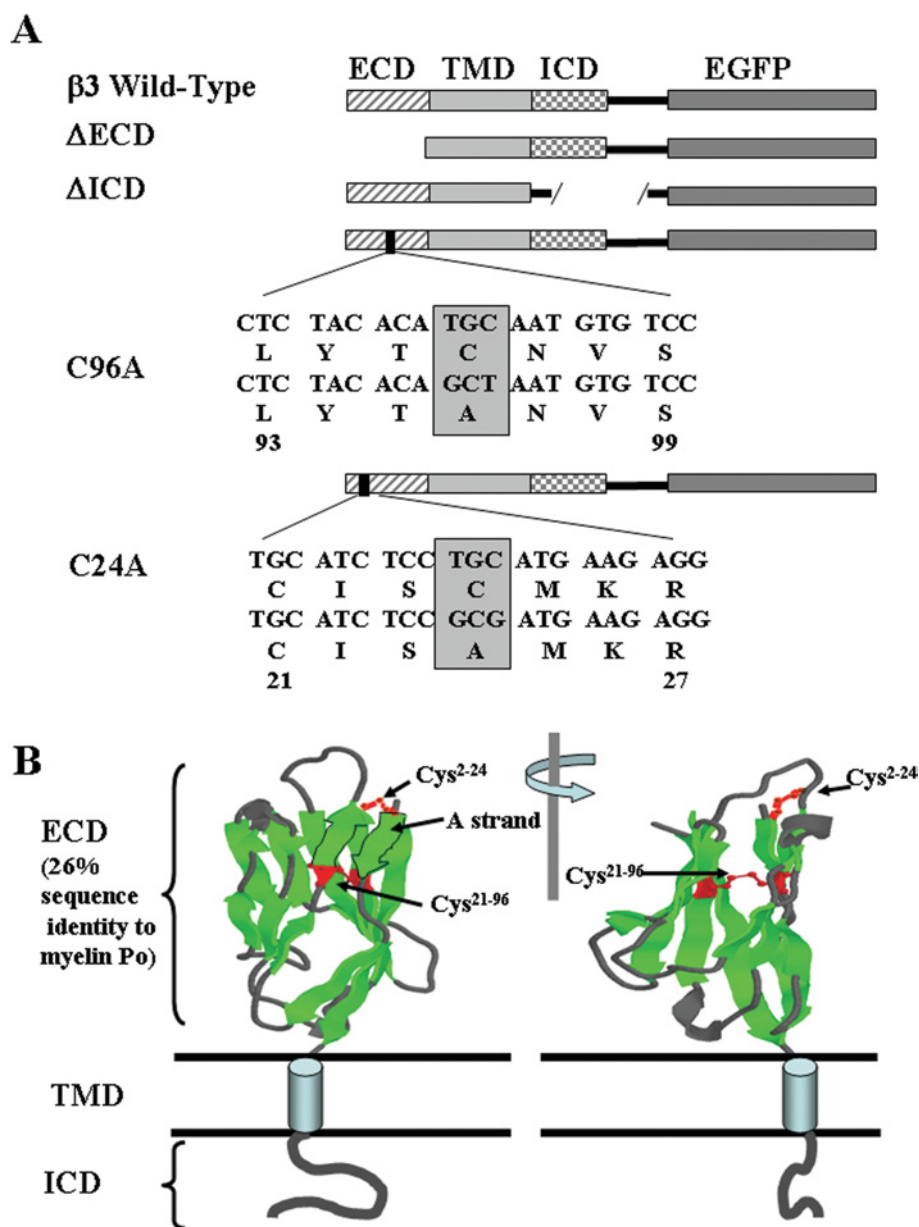


Figure 1 Summary of the mutant constructs

(A) Diagrammatic representation of the EGFP-tagged $\beta 3$ constructs showing the relative positions of the ECD, TMD and ICD. The Δ ECD mutant had amino acids 1–135 removed. The Δ ICD mutant had amino acids 158–191 removed. Point mutations C24A and C96A in the ECD are indicated. (B) Modelling of the V-type Ig fold of the ECD. The representation is based on the known structure of myelin P₀, a protein showing approx. 26% amino acid sequence identity with the ECD of $\beta 3$ [9] and for which an accurate structure is known [25]. The model shows the proposed location and disulphide bonds for Cys²–Cys²⁴ and Cys²¹–Cys⁹⁶.

above. All constructs were verified by sequencing of both sense and anti-sense strands. Plasmids were amplified in *Escherichia coli* strain DH5 α and purified with a Qiagen Plasmid Maxi kit.

Cell culture

PC12 cells were obtained from the A.T.C.C. (Manassas, VA, U.S.A.). In the present study, we used two separate CHO (Chinese-hamster ovary) cell lines: CHO-K1 cells (obtained from the European Collection of Cell Cultures, Porton Down, Salisbury, Wilts., U.K.) and CHO-K1 cells stably expressing the cardiac α -subunit Na_v1.5 sodium channel (hereafter referred to as CHO-K1/1.5 cells) [16]. All cells were grown at 37°C in a humidified

atmosphere with 5% CO₂. The PC12 cells were cultured in Kaighn's modification of Ham's F12 medium (Gibco, Paisley, U.K.), supplemented with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 15% (v/v) horse serum and 2.5% (v/v) foetal bovine serum. The CHO cell lines were grown in DMEM (Dulbecco's modified Eagle's medium)/F12 mixture (Invitrogen) supplemented with 10% foetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μ g/ml). Media for the CHO-K1/1.5 cells were supplemented with G418 (500 μ g/ml; Sigma).

Immunocytochemistry

Cells were seeded on to borosilicate glass coverslips (BDH) coated with poly(L-lysine) (Sigma) and grown to confluency

before being transiently transfected with 1 μg of DNA using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h, cells were fixed in either 4% (w/v) paraformaldehyde or -20°C methanol and then washed in PBS and permeabilized for 30 min in a blocking buffer containing PBS, 1% BSA and 0.03% Triton X-100. Cells were incubated with primary antibodies diluted in the blocking buffer in a humidified chamber overnight at 4°C . Cells were then washed with PBS before incubating with fluorescent-conjugated secondary antibodies diluted in the blocking buffer. Cells were washed with PBS and water, nuclear stained with Hoescht 33342 and then mounted on to coverslips using Vectashield mounting medium (Vector Laboratories). Anti-mouse *cis*-Golgi marker GM130, anti-rat KDEL antibody, Cy3-labelled goat anti-mouse antibody and Cy3-labelled goat anti-rabbit antibody were generously provided by Dr Edward Bampton (University of Cambridge, Cambridge, U.K.). Confocal imaging was performed with a PerkinElmer Ultraview LCI system using an inverted Olympus IX70 microscope. EGFP excitation was at 488 nm using an argon ion laser (Melles Griot) and emission signals were obtained with a 508 nm emission filter. Cy3 excitation was at 568 nm and emission signals were obtained with a 600 nm emission filter. All image analyses were performed using the Ultraview software program.

Patch clamp electrophysiology

Wild-type and mutant $\beta 3$ constructs lacking EGFP were engineered into the vector pcDNA3.1 (Invitrogen) and transiently transfected into the CHO-K1/1.5 cells (10 μg of DNA) together with CD8 (0.2 μg of DNA) using Lipofectamine™ 2000. Transfected cells were identified 2 days later by immunobeads (CD8-Dynabeads; Dynal, Oslo, Norway). Cells with > 5 beads attached were selected for recording. Sodium currents were recorded using the whole-cell configuration of the patch clamp recording technique with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, U.S.A.). All voltage methods were applied using pCLAMP 8 software (Axon Instruments) and a Digidata 1322A (Axon Instruments). Currents were amplified and low-pass filtered (2 kHz) and sampled at 33 kHz. Borosilicate glass pipettes (0.6–1.0 M Ω) were filled with 130 mM CsCl, 1 mM MgCl₂, 5 mM MgATP²⁻, 10 mM BAPTA (bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid) and 5 mM Hepes (pH adjusted to 7.4 with CsOH). Cells were plated on glass coverslips and superfused with 130 mM NaCl, 4 mM KCl, 1 mM CaCl₂, 5 mM MgCl₂, 5 mM Hepes and 5 mM glucose (pH adjusted to 7.4 with NaOH). Initial whole-cell patch clamp resistance was 1–1.5 M Ω and was further reduced by a series resistance compensation of 75%. Cells were held at -80 mV for 7 min to allow equilibrium gating to reach steady state. Data from cells with a current amplitude of > 8 nA, or from cells that showed evidence of poor voltage control as reflected by the shape of the current–voltage curve, were excluded from the study. All experiments were performed at room temperature (20–22°C). Current–voltage relationship was determined with a 20 ms voltage pulse from -80 to $+60$ mV in steps of 5 mV from a holding potential of -120 mV. Steady-state activation was derived from the current–voltage relationship using the equation:

$$g = I(V - V_{\text{rev}}) \quad (1)$$

where g is the conductance, I is the peak current amplitude, V is the test potential and V_{rev} is the measured reversal potential. Steady-state inactivation was determined using a two-pulse method, from a holding potential of -120 mV and test potentials between -130 and -20 mV for 1000 ms followed by a test pulse to $+10$ mV.

Equilibrium gating plots were fitted to the Boltzmann function:

$$g = 1 \{ 1 + \exp[(V - V_{1/2})/k] \} \quad (2)$$

where g is the conductance, $V_{1/2}$ is the voltage of half-maximal activation or inactivation and k is the slope factor.

Data analysis

Data analysis was performed using Clampfit software (V8; Axon Instruments), Origin (V5; Microcal Software, Northampton, MA, U.S.A.), Sigmaplot (Jandel Scientific, Madera, CA, U.S.A.) and Microsoft Excel. Standard one-way ANOVA followed by Tukey's post-hoc test was used to determine significance of differences. Averaged data are presented as means \pm S.E.M. Values of $P < 0.05$ were considered as statistically significant.

RESULTS

The $\beta 3$ -subunit was first isolated from the rat pheochromocytoma cell line PC12 [9]. To study the intracellular targeting of $\beta 3$ in its most physiologically normal cellular environment, we first examined the distribution of wild-type and mutant $\beta 3$ in this cell line. PC12 cells express Na_v1.2 α -subunit [17,18] and, in addition to $\beta 3$, also express the $\beta 1$ auxiliary subunit [9]. PC12 cells respond to NGF (nerve growth factor) by differentiating into a more neuronal phenotype with a concomitant increase in the expression of sodium channel α -subunits [19]. Undifferentiated PC12 cells express heterogeneity in the level of their α -subunit expression, with some cells expressing only low levels [20]. We therefore investigated the level of α -subunit by Western blotting. There was detectable expression of α -subunit under non-NGF-treated conditions in the line of undifferentiated PC12 used in these experiments, and NGF treatment did not affect either the subcellular distribution of $\beta 3$ described below or the level of transfected $\beta 3$ expression (results not shown). To confirm that the targeting behaviour was not primarily dependent on cell type or α -subunit isoform, we also examined the distribution of wild-type and mutant $\beta 3$ in CHO-K1/1.5 cells (see the Materials and methods section) [16].

We constructed $\beta 3$ chimaeras tagged via a short C-terminal linker region to EGFP (Figures 1A and 1B) and expressed them by transient transfection. We first confirmed that the addition of EGFP-tagged to wild-type $\beta 3$ did not modify the electrophysiological properties of associated Na_v1.5 sodium channels (results not shown). This is consistent with previous work showing that addition of GFP to the C-terminus of $\beta 1$ does not adversely affect channel gating properties [21]. In both cell lines, EGFP-tagged wild-type $\beta 3$ labelled the plasma membrane, together with a variable degree of intracellular staining consistent with elements of the secretory pathway (Figures 2A and 2F). The $\beta 3$ -subunit contains a single N-terminal ECD, which adopts a V-type Ig (immunoglobulin) fold, a single α -helical TMD (transmembrane domain) and a small C-terminal ICD [9]. To examine the significance of the ECD and ICD in channel assembly and subunit targeting, we generated separate $\beta 3$ constructs completely lacking each of them (Figure 1). These constructs retained both an N-terminal signal sequence and TMD, thus allowing efficient targeting to the membrane of the ER (endoplasmic reticulum) after translation. In PC12 and CHO-K1/1.5 cells, the ΔECD $\beta 3$ mutant showed internal staining consistent with compartments along the secretory pathway together with some plasma-membrane staining (Figures 2B and 2G). In striking contrast, the EGFP-tagged ΔICD $\beta 3$ mutant showed no evidence of surface staining but labelled an internal highly reticulated compartment that suggests

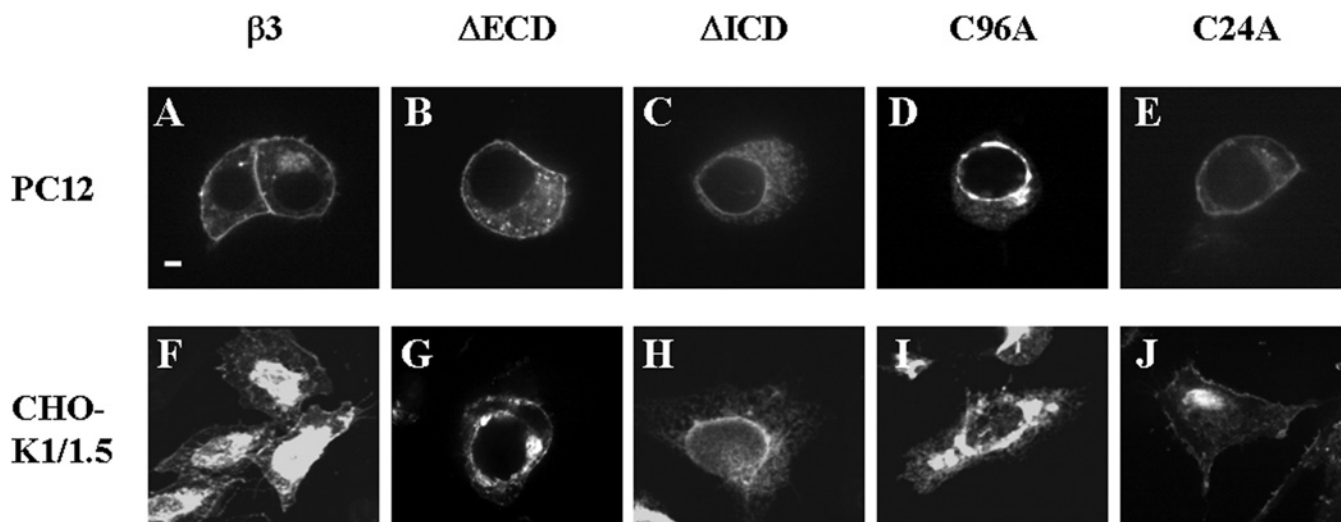


Figure 2 Expression of EGFP-tagged $\beta 3$ constructs in PC12 and CHO-K1/1.5 cells

(A–E) PC12 cells and (F–J) CHO-K1/1.5 cells were fixed 24 h after transient transfection with the indicated constructs. Each is typical of at least five independent experiments. (A, F) Wild-type $\beta 3$. (B, G) The Δ ECD mutant. (C, H) The Δ ICD mutant. (D, I) The C96A mutant. (E, J) The C24A mutant. Wild-type $\beta 3$ and Δ ECD and C24A mutants exhibit some plasma-membrane staining. The Δ ICD and C96A mutants failed to reach the plasma membrane and were retained inside the cell. Scale bar, 2 μ m.

ER (Figures 2C and 2H). Double labelling with an antibody raised against the KDEL sequence of luminal ER proteins [22] showed some co-localization, although there were also non-overlapping areas (Figures 3A–3C). Importantly, however, there was no co-localization of the Δ ICD $\beta 3$ mutant with the *cis*-Golgi marker GM130 [23] (Figures 3D–3F), suggesting that the mutant did not reach the early compartments of the Golgi. A similar pattern has been noted for other transiently transfected mutant proteins that cannot exit the ER. In such cases, the mutant protein often accumulates in ER subcompartments such as Russell bodies that are denuded of soluble ER markers [24].

We have previously suggested a model for the ECD of $\beta 3$ based on the known three-dimensional structure of the Ig fold of myelin P₀ with which it shares approx. 26% sequence identity [9]. A critical feature of this model is the disulphide bond between the cysteine residues Cys⁹⁶ and Cys²¹ (Figure 1). This is an absolutely conserved feature of all proteins containing an Ig fold and is likely to play a critical role in subunit stability [9,25]. To investigate the importance of the disulphide bond, we mutated Cys⁹⁶ to an alanine residue (Figure 1). The EGFP-tagged C96A mutant showed a complete absence from the plasma membrane (Figures 2D and 2I) and a dramatic intracellular staining largely of the perinuclear region showing partial co-localization with KDEL proteins (Figures 3G–3I), but no co-localization with GM130 (Figures 3J–3L), a result similar to the pattern shown by the Δ ICD $\beta 3$ mutant. The ECD of $\beta 3$ contains two further cysteine residues Cys² and Cys²⁴ (Figure 1). On the basis of their mutual proximity in the proposed $\beta 3$ ECD structure, we have previously suggested that these may form a second disulphide bond [9]. This feature is present in both $\beta 3$ [9] and $\beta 1$ [26], but is not found on other proteins with Ig folds, suggesting a functional importance unique to sodium channel β -subunits. Nevertheless, the C24A mutation was much less disruptive than the $\beta 3$ C96A mutation, since it was successfully delivered to the plasma membrane in both PC12 and CHO-K1/1.5 cells (Figures 2E and 2J).

These results raise the question of whether the $\beta 3$ -subunit alone can be correctly targeted or whether it must travel to the plasma membrane in association with the α -subunit. To address this question, we separately expressed wild-type and mutant $\beta 3$

constructs in the CHO-K1 cell line from which CHO-K1/1.5 was originally constructed and which lacks significant endogenous α -subunit expression [16]. Surprisingly, wild-type $\beta 3$ alone was correctly targeted to the plasma membrane under these conditions (Figure 4A). Furthermore, the targeting behaviour of the mutant $\beta 3$ constructs was largely identical with that observed in PC12 and CHO-K1/1.5 cells in that the C24A mutant was correctly targeted to the plasma membrane (Figure 4E), but the Δ ICD and C96A $\beta 3$ mutants were not (Figures 4C and 4D). There was, however, one notable difference from the PC12 and CHO-K1/1.5 results. When expressed alone in CHO-K1 cells, the Δ ECD $\beta 3$ mutant failed to reach the plasma membrane (Figure 4B).

We next expanded these studies to compare the effect of wild-type or mutant $\beta 3$ on sodium channel gating. In view of the fact that PC12 cells contain endogenous $\beta 1$ - and $\beta 3$ -subunits, and that these auxiliary subunits affect channel gating parameters differently [9,16], the CHO-K1/1.5 cell line was used for electrophysiological evaluation. These cells lack endogenous β -subunits [27]. The Na_v1.5 α -subunit has been previously shown to be modulated by $\beta 3$ co-expression [12,16]. In agreement with previous findings [16], co-expression with wild-type $\beta 3$ significantly ($P < 0.001$) shifted both the voltage of half-maximum inactivation and activation ($V_{1/2}$) to more hyperpolarized potentials (Figures 5A and 5B and Table 1). In contrast, co-expression of Δ ECD $\beta 3$ mutant with Na_v1.5 resulted in a $V_{1/2}$ for steady-state inactivation that was not significantly different from Na_v1.5 alone. The most profound effect of the Δ ECD $\beta 3$ mutant was on sodium channel activation gating, significantly ($P < 0.001$) shifting $V_{1/2}$ values to greater depolarized voltages than that recorded for Na_v1.5 alone (Figure 5A and Table 1). In the cases of both Δ ICD and the C96A $\beta 3$ mutants, co-expression with Na_v1.5 abolished the shift in steady-state inactivation to $V_{1/2}$ values that were no different from Na_v1.5 alone and attenuated the modulation of activation gating observed in cells co-expressed with $\beta 3$ (Figures 5A and 5B and Table 1). In the case of the C24A mutant, there were no adverse effects on steady-state activation gating with similar $V_{1/2}$ values to that recorded for $\beta 3$ alone. However, steady-state inactivation gating was significantly attenuated ($P < 0.001$) when compared with $\beta 3$ co-expression,

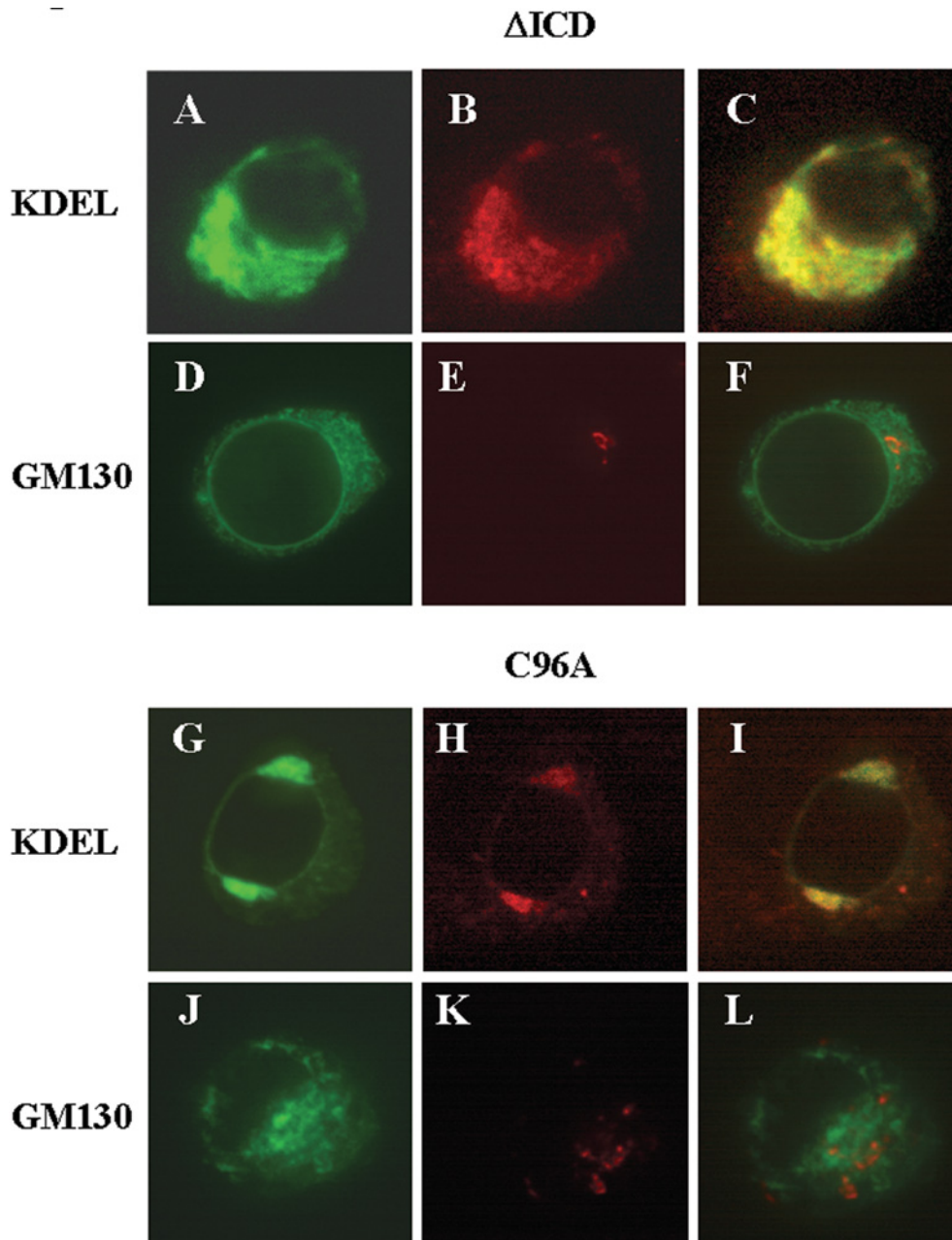


Figure 3 Intracellular localization of Δ ICD and C96A mutants

(A–F) The EGFP-tagged $\beta 3$ Δ ICD and (G–L) the EGFP-tagged C96A mutants were transiently transfected into PC12 cells. (A, D, G, J) EGFP staining. (B, H) Cells co-stained with Cy3-labelled antibodies (red) raised against KDEL, a marker for soluble proteins of the ER. (E, K) Cells co-stained with Cy3-labelled antibodies (red) raised against GM130, a marker for the *cis*-Golgi network. (C, F, I, L) Merged images.

reaching $V_{1/2}$ values that were no longer significantly different from $\text{Na}_v 1.5$ alone (Figure 5B and Table 1). In all the experiments, slope factors (k ; Table 1) and peak current amplitudes were not changed from $\text{Na}_v 1.5$ alone (results not shown).

DISCUSSION

Our results reveal the importance of the ECD and ICD of the $\beta 3$ -subunit in modulating the electrophysiological properties of the sodium channel and in the efficient transport of the $\beta 3$ -subunit to the plasma membrane. As detected by enhanced peak current amplitude, the effects of β -subunit co-expression on the level of

functional sodium channels in the plasma membrane are unclear. For example, in *Xenopus* oocytes, co-expression of either $\beta 1$ or $\beta 3$ with $\text{Na}_v 1.5$ leads to an increase in current amplitude and is interpreted to suggest that $\beta 1$ and $\beta 3$ increase the efficiency of channel export from the ER [12]. However, in the same expression system, $\beta 1$ or $\beta 3$ co-expression with the neuronal sodium channel isoforms, $\text{Na}_v 1.2$ and $\text{Na}_v 1.3$, had no effect on current amplitude [9,28]. Similarly, differential effects of β -subunit co-expression on current amplitudes have also been reported using mammalian expression systems [14,16,27]. The reason for this discrepancy is not clear and may reflect differences in sodium channel behaviour between these two rather different expression systems.

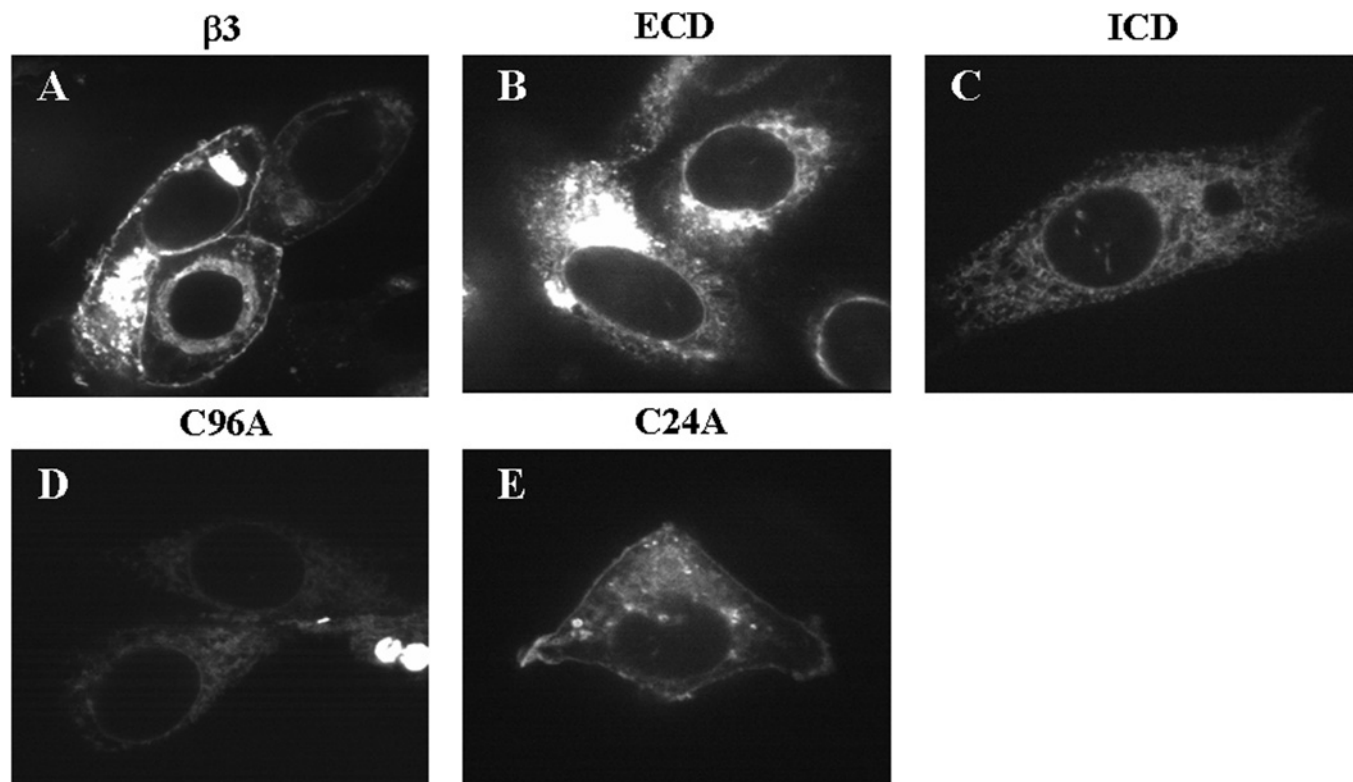


Figure 4 Expression of EGFP-tagged $\beta 3$ constructs in CHO-K1 cells

(A) Wild-type $\beta 3$. (B) The Δ ECD mutant. (C) The Δ ICD mutant. (D) The C96A mutant. (E) The C24A mutant. Cells were fixed 24 h after transient transfection with the indicated constructs. Each is typical of at least five independent experiments. Wild-type $\beta 3$ and the C24A mutation showed plasma-membrane staining. The Δ ECD, Δ ICD and C96A mutations did not reach the surface.

For example, efficient $\beta 3$ -dependent retention of channels at the plasma membrane may be operating in oocytes. It has been noted that sequences in the cytoplasmic domain of $\beta 1$ modulate stable interactions of the plasma-membrane-localized sodium channel and cytoskeletal components [13]. In the present study, neither wild-type $\beta 3$ nor any of the $\beta 3$ mutations changed peak current amplitude of $\text{Na}_v 1.5$ currents. In the case of $\beta 1$, it has been suggested that association with the α -subunit occurs in the ER and this may enhance the export efficiency of the channel [21]. However, our analysis suggests that transport of α -subunits to the plasma membrane has no obligatory requirement for $\beta 3$ in any mammalian cell line expression system, and since export of wild-type $\beta 3$ can occur independently of the α -subunit, functional association between wild-type $\beta 3$ - and α -subunits may occur at the plasma membrane as well as the ER. Recently, Adachi et al. [29] have reported that expression of $\beta 3$ alone in the Saos2 human cancer cell line led to the accumulation of $\beta 3$ in the ER which induced apoptosis. We did not observe any evidence for enhanced cell death in cells transfected with $\beta 3$ alone, suggesting that the induction of apoptosis may be a cell-specific trait. However, Adachi et al. [29] did observe some $\beta 3$ on the plasma membrane of Saos2 cells, consistent with our findings.

Deletion of the ECD did not prevent the trafficking of the construct to the surface in PC12 or in CHO-K1/1.5 cells. However, the Δ ECD $\beta 3$ mutant was retained intracellularly when expressed in CHO-K1 cells without an accompanying α -subunit. We suggest that this can best be explained if wild-type $\beta 3$ can be exported to the plasma membrane by either of two pathways: together with the α -subunit or alone; however, deletion of the ECD blocks this second pathway, so its export is now dependent on the presence of the α -subunit. Intracellular retention of the Δ ECD $\beta 3$ mutant

in cells not co-expressing an α -subunit partner raises further questions about the nature of the retention mechanism. A major quality control pathway by which incorrectly folded proteins are retained and ultimately eliminated involves the activity of ER lectins that bind to the N-linked glycans post-translationally added to the luminal sequences of proteins [30–32], but these glycans are presumably missing in the Δ ECD $\beta 3$ mutant. Relatively little is known about glycan-independent quality control within the secretory pathway [33], and the EGFP-tagged Δ ECD mutant may be a useful model with which to study this mechanism.

When co-expressed with $\text{Na}_v 1.5$, the Δ ECD $\beta 3$ mutant resulted in the complete abolition of the steady-state inactivation gating shifts observed on co-expressing the wild-type $\beta 3$. These findings suggest that the ECD of $\beta 3$ is essential for the sodium channel equilibrium gating effects, as has been previously suggested for the $\beta 1$ -subunit [26]. Interestingly, steady-state activation parameters were shifted to greater depolarized potentials than $\text{Na}_v 1.5$ alone, implying a continued association of the $\beta 3$ ICD with the channel, even in the absence of an ECD. This view is consistent with a growing body of evidence that implicates the ICD of β -subunits as the major factor in binding sodium channel α -subunits. For example, a recently identified point mutation in the ICD of $\text{Na}_v 1.1$ has been described that interferes with binding to the ICD of $\beta 1$ and causes epilepsy [34]. Similarly, mutational studies show that the deletion of the ICD of $\beta 1$ largely abolished both the functional modulation of the $\text{Na}_v 1.2$ subunit by $\beta 1$, and $\beta 1/\alpha$ -subunit binding [14]. Furthermore, yeast two-hybrid results indicate that the ICD of $\beta 1$ and $\beta 3$ can bind the cytoplasmic domain of $\text{Na}_v 1.1$ α -subunit [34]. The Δ ICD $\beta 3$ mutant was retained intracellularly, and the functional data from the Δ ICD mutant expressed in CHO-K1/1.5 cells were fully consistent with

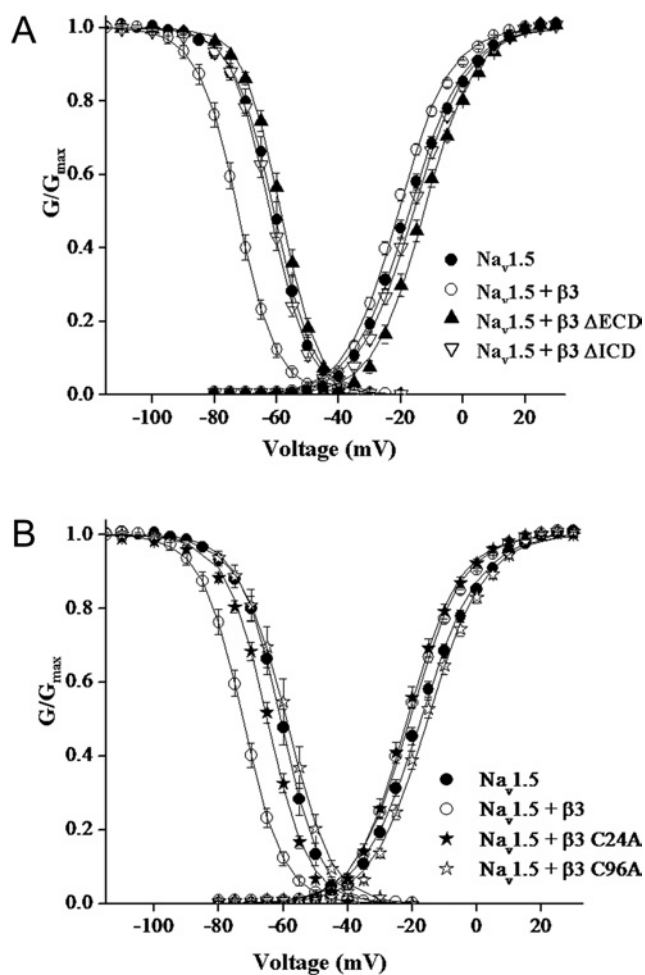


Figure 5 Steady-state inactivation and activation plots

(A) ●, $\text{Na}_v1.5$; ○, $\text{Na}_v1.5 + \beta 3$; ▲, $\text{Na}_v1.5 + \beta 3 \Delta\text{ECD}$; and ▽, $\text{Na}_v1.5 + \beta 3 \Delta\text{ICD}$. (B) ●, $\text{Na}_v1.5$; ○, $\text{Na}_v1.5 + \beta 3$; ★, $\text{Na}_v1.5 + \beta 3 \text{C}24\text{A}$; and ☆, $\text{Na}_v1.5 + \beta 3 \text{C}96\text{A}$. Voltage dependence of activation was determined with a 20 ms voltage pulse from -80 to $+60$ mV in steps of 5 mV from a holding potential of -120 mV. Steady-state activation was derived from eqn (1). Steady-state inactivation was determined using a two-pulse method, from a holding potential of -120 mV and test potentials between -130 and -20 mV for 1 s followed by a test pulse to $+10$ mV. The data points show means \pm S.E.M. for 13–27 cells. The solid line represents the average of each Boltzmann function fit to the individual data curves. The steady-state parameters are shown in Table 1.

its absence from the plasma membrane. This suggests a possible additional role for the ICD in the correct targeting of the $\beta 3$ -subunit, most likely as a result of sequence motifs which would normally allow incorporation into COPII (coatamer protein II)-coated vesicles [35]. If, as we suggest above, the α - and $\beta 3$ -subunits can interact in the ER, the continued normal expression of α -subunits at the plasma membrane of ΔICD $\beta 3$ mutant-expressing cells implies that the ΔICD mutant can no longer bind strongly to the α -subunit. Otherwise, a proportion of the assembled channel would be expected to be retained in the ER along with the ΔICD $\beta 3$ mutant. This inference is consistent with the evidence noted above for a major role of the ICD in α -subunit binding.

The C96A mutant abolished the critical intramolecular disulphide bond that is believed to hold together the two faces of the ECD within the Ig fold. The mutant was efficiently retained intracellularly. The electrophysiological results for the C96A mutant expressed with $\text{Na}_v1.5$ was indistinguishable from $\text{Na}_v1.5$

Table 1 Effects of $\beta 3$ -subunit deletions and mutations on equilibrium gating parameters

Parameters are calculated from the Boltzmann function (eqn 2) for data shown in Figures 5(A) and 5(B). Values represent means \pm S.E.M.

	Steady-state inactivation			Steady-state activation		
	$V_{1/2}$ (mV)	k (mV)	n	$V_{1/2}$ (mV)	k (mV)	n
$\text{Na}_v1.5$	$-61.1 \pm 1.3^*$	5.7 ± 0.3	17	$-17.4 \pm 0.8^*$	-8.7 ± 0.2	27
$\text{Na}_v1.5 + \beta 3$	$-72.6 \pm 1.0^\dagger$	5.9 ± 0.2	21	$-20.7 \pm 0.5^\dagger$	-8.2 ± 0.2	15
$\text{Na}_v1.5 + \beta 3 \Delta\text{ECD}$	$-58.7 \pm 0.9^*$	5.4 ± 0.1	26	$-12.5 \pm 0.8^\dagger$	-7.8 ± 0.1	13
$\text{Na}_v1.5 + \beta 3 \Delta\text{ICD}$	$-62.1 \pm 0.9^*$	5.7 ± 0.1	22	$-15.8 \pm 0.8^*$	-8.5 ± 0.1	26
$\text{Na}_v1.5 + \beta 3 \text{C}96\text{A}$	$-59.5 \pm 1.7^*$	6.2 ± 0.2	14	$-15.1 \pm 0.9^*$	-8.4 ± 0.2	15
$\text{Na}_v1.5 + \beta 3 \text{C}24\text{A}$	$-65.1 \pm 0.7^*$	6.2 ± 0.2	23	$-21.1 \pm 1.1^\dagger$	-7.6 ± 0.2	19

* $P < 0.001$ for comparison with $\beta 3$.

† $P < 0.001$ for comparison with $\text{Na}_v1.5$.

alone, consistent with a plasma-membrane-localized α -subunit that is largely unassociated with $\beta 3$. The intracellular retention of the $\beta 3$ -subunit with this single amino acid mutation is typical of proteins that fail to fold correctly during secretion [32]. We suggest that by preventing the formation of the disulphide bond, the C96A mutant destabilizes the folding of the entire ECD of $\beta 3$ and leads to its retention by the ER quality control machinery. In $\beta 1$, a point mutation that converts Cys²¹ into a tryptophan residue disrupts the equivalent disulphide bond and has been shown to cause an inherited form of febrile epilepsy. This mutant also fails to modulate α -subunit kinetics [36]. However, in contrast with our results for $\beta 3$, the C21W mutant in $\beta 1$ does reach the plasma membrane [37]. The reasons for the difference are not clear. In a protein with a typical Ig fold, the disulphide bond equivalent to Cys²⁴–Cys⁹⁶ hydrophobically stacks on top of a highly conserved tryptophan residue (Trp³⁸ in $\beta 3$) [9]. Tryptophan is a much more hydrophobic amino acid than alanine. Hence it is possible that even without the stabilizing disulphide bond, the C21W mutation of $\beta 1$ may allow the two hydrophobic faces of the Ig fold and thus maintain enough partial structure via hydrophobic stacking of the two tryptophan residues to avoid retention in the ER.

Deletion of Cys²⁴ did not affect α -subunit binding or trafficking to the surface, suggesting little or no effect on folding efficiency. The mutation did, however, significantly attenuate the shift in steady-state inactivation gating parameters observed with co-expression of $\beta 3$, and showed no effect on steady-state activation parameters. We have previously suggested that this cysteine residue may form a disulphide with Cys² and thereby stabilize the first ribbon of β -pleated sheet (the A strand in Figure 1B) which may be required for modulating the α -subunit [9]. On the α -subunit, the proposed interaction site for the $\beta 1$ -subunit is within the extracellular SS1–SS2 loop between segments 5 and 6 of domain IV [38,39]. Previous studies have suggested that the extracellular loops are important determinants of sodium channel gating. In addition, domain IV, with particular emphasis on the S4 segment, is thought to play an important role in sodium channel inactivation [39]. Disruption of the Cys²–Cys²⁴ disulphide bond could cause a subtle perturbation of this interaction without disrupting the more extensive $\beta 3/\alpha$ -subunit binding of the ICD. This would explain the effects on inactivation gating kinetics and support a role for the Cys²–Cys²⁴ disulphide bond in optimizing the interaction with the α -subunit.

In summary, our results integrate a structural, cell biological and electrophysiological approach to the role of the $\beta 3$ -subunit. They emphasize that these factors may be functionally interrelated and that distinct domains on $\beta 3$ play multiple roles in the targeting and modulation of gating properties.

We thank Dr Augustus O. Grant (Duke University Medical Center, Durham, NC, U.S.A.) for the gift of the CHO-K1/1.5 cell line. We thank Dr Edward Bampton and Dr Christoph Goemans (University of Cambridge, Cambridge, U.K.) and the Cardiovascular Research Center (University of Virginia, Charlottesville, VA, U.S.A.) for technical advice on confocal microscopy. We thank Dr Sarah Lummis (Department of Biochemistry, University of Cambridge, Cambridge, U.K.) and Dr Jamie Vandenberg (Victor Chang Cardiac Research Institute, Darlinghurst, NSW, Australia) for helpful discussions and comments on this paper. This work was supported by grants from the Wellcome Trust (to A.P.J.), the American Heart Association Virginia Affiliate (to M.K.P.) and the Korean Science and Engineering Foundation (to S.-H.K.).

REFERENCES

- Yu, F. H. and Catterall, W. A. (2003) Overview of the voltage-gated sodium channel family. *Genome Biol.* **4**, 207
- Goldin, A. L. (2001) Resurgence of sodium channel research. *Annu. Rev. Physiol.* **63**, 871–894
- Taylor, C. P. and Meldrum, B. S. (1995) Na⁺ channels as targets for neuroprotective drugs. *Trends Pharmacol. Sci.* **16**, 309–316
- Cannon, S. C. (1996) Sodium channel defects in myotonia and periodic paralysis. *Annu. Rev. Neurosci.* **19**, 141–164
- Keating, M. T. and Sanguinetti, M. C. (2001) Molecular and cellular mechanisms of cardiac arrhythmias. *Cell (Cambridge, Mass.)* **104**, 569–580
- Lossin, C., Wang, D. W., Rhodes, T. H., Vanoye, C. G. and George, Jr, A. L. (2002) Molecular basis of an inherited epilepsy. *Neuron* **34**, 877–884
- Isom, L. L., De Jongh, K. S., Patton, D. E., Reber, B. F., Offord, J., Charbonneau, H., Walsh, K., Goldin, A. L. and Catterall, W. A. (1992) Primary structure and functional expression of the beta 1 subunit of the rat brain sodium channel. *Science* **256**, 839–842
- Isom, L. L., Ragsdale, D. S., De Jongh, K. S., Westenbroek, R. E., Reber, B. F., Scheuer, T. and Catterall, W. A. (1995) Structure and function of the beta 2 subunit of brain sodium channels, a transmembrane glycoprotein with a CAM motif. *Cell (Cambridge, Mass.)* **83**, 433–442
- Morgan, K., Stevens, E. B., Shah, B., Cox, P. J., Dixon, A. K., Lee, K., Pinnock, R. D., Hughes, J., Richardson, P. J., Mizuguchi, K. et al. (2000) Beta 3: an additional auxiliary subunit of the voltage-sensitive sodium channel that modulates channel gating with distinct kinetics. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 2308–2313
- Yu, F. H., Westenbroek, R. E., Silos-Santiago, I., McCormick, K. A., Lawson, D., Ge, P., Ferriera, H., Lilly, J., DiStefano, P. S., Catterall, W. A. et al. (2003) Sodium channel beta4, a new disulfide-linked auxiliary subunit with similarity to beta2. *J. Neurosci.* **23**, 7577–7585
- Stevens, E. B., Cox, P. J., Shah, B. S., Dixon, A. K., Richardson, P. J., Pinnock, R. D. and Lee, K. (2001) Tissue distribution and functional expression of the human voltage-gated sodium channel beta3 subunit. *Pflügers Arch.* **441**, 481–488
- Fahmi, A. I., Patel, M., Stevens, E. B., Fowden, A. L., John, III, J. E., Lee, K., Pinnock, R., Morgan, K., Jackson, A. P. and Vandenberg, J. I. (2001) The sodium channel beta-subunit SCN3b modulates the kinetics of SCN5a and is expressed heterogeneously in sheep heart. *J. Physiol. (Cambridge, U.K.)* **537**, 693–700
- McEwen, D. P., Meadows, L. S., Chen, C., Thyagarajan, V. and Isom, L. L. (2004) Sodium channel beta1 subunit-mediated modulation of Nav1.2 currents and cell surface density is dependent on interactions with contactin and ankyrin. *J. Biol. Chem.* **279**, 16044–16049
- Meadows, L., Malhotra, J. D., Stetzer, A., Isom, L. L. and Ragsdale, D. S. (2001) The intracellular segment of the sodium channel beta 1 subunit is required for its efficient association with the channel alpha subunit. *J. Neurochem.* **76**, 1871–1878
- Zimmer, T. and Benndorf, K. (2002) The human heart and rat brain IIA Na⁺ channels interact with different molecular regions of the beta1 subunit. *J. Gen. Physiol.* **120**, 887–895
- Ko, S. H., Lenkowski, P. W., Lee, H. C., Mounsey, J. P. and Patel, M. K. (2005) Modulation of Na(v)1.5 by beta1- and beta3-subunit co-expression in mammalian cells. *Pflügers Arch.* **449**, 403–412
- Mandel, G., Cooperman, S. S., Maue, R. A., Goodman, R. H. and Brehm, P. (1988) Selective induction of brain type II Na⁺ channels by nerve growth factor. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 924–928
- D'Arcangelo, G., Paradiso, K., Shepherd, D., Brehm, P., Halegoua, S. and Mandel, G. (1993) Neuronal growth factor regulation of two different sodium channel types through distinct signal transduction pathways. *J. Cell Biol.* **122**, 915–921
- Rudy, B., Kirschenbaum, B. and Greene, L. A. (1982) Nerve growth factor-induced increase in saxitoxin binding to rat PC12 pheochromocytoma cells. *J. Neurosci.* **2**, 1405–1411
- Pollack, J. D., Kremplin, M. and Rudy, B. (1990) Differential effects of NGF, FGF, EGF, cAMP, and dexamethasone on neurite outgrowth and sodium channel expression in PC12 cells. *J. Neurosci.* **10**, 2626–2637
- Zimmer, T., Biskup, C., Bollensdorff, C. and Benndorf, K. (2002) The beta1 subunit but not the beta2 subunit colocalizes with the human heart Na⁺ channel (hH1) already within the endoplasmic reticulum. *J. Membr. Biol.* **186**, 13–21
- Munro, S. and Pelham, H. R. (1987) A C-terminal signal prevents secretion of luminal ER proteins. *Cell (Cambridge, Mass.)* **48**, 899–907
- Nakamura, N., Rabouille, C., Watson, R., Nilsson, T., Hui, N., Slusarewicz, P., Kreis, T. E. and Warren, G. (1995) Characterization of a cis-Golgi matrix protein, GM130. *J. Cell Biol.* **131**, 1715–1726
- Kopito, R. R. and Sitia, R. (2000) Aggresomes and Russell bodies. Symptoms of cellular indigestion? *EMBO Rep.* **1**, 225–231
- Shapiro, L., Doyle, J. P., Hensley, P., Colman, D. R. and Hendrickson, W. A. (1996) Crystal structure of the extracellular domain from P0, the major structural protein of peripheral nerve myelin. *Neuron* **17**, 435–449
- McCormick, K. A., Isom, L. L., Ragsdale, D., Smith, D., Scheuer, T. and Catterall, W. A. (1998) Molecular determinants of Na⁺ channel function in the extracellular domain of the beta1 subunit. *J. Biol. Chem.* **273**, 3954–3962
- Meadows, L. S., Chen, Y. H., Powell, A. J., Clare, J. J. and Ragsdale, D. S. (2002) Functional modulation of human brain Nav1.3 sodium channels, expressed in mammalian cells, by auxiliary beta 1, beta 2 and beta 3 subunits. *Neuroscience* **114**, 745–753
- Lenkowski, P. W., Shah, B. S., Dinn, A. E., Lee, K. and Patel, M. K. (2003) Lidocaine block of neonatal Nav1.3 is differentially modulated by co-expression of beta1 and beta3 subunits. *Eur. J. Pharmacol.* **467**, 23–30
- Adachi, K., Toyota, M., Sasaki, Y., Yamashita, T., Ishida, S., Ohe-Toyota, M., Maruyama, R., Hinoda, Y., Saito, T., Imai, K. et al. (2004) Identification of SCN3B as a novel p53-inducible proapoptotic gene. *Oncogene* **23**, 7791–7798
- Chevet, E., Cameron, P. H., Pelletier, M. F., Thomas, D. Y. and Bergeron, J. J. (2001) The endoplasmic reticulum: integration of protein folding, quality control, signaling and degradation. *Curr. Opin. Struct. Biol.* **11**, 120–124
- Cabral, C. M., Liu, Y. and Sifers, R. N. (2001) Dissecting glycoprotein quality control in the secretory pathway. *Trends Biochem. Sci.* **26**, 619–624
- Kleizen, B. and Braakman, I. (2004) Protein folding and quality control in the endoplasmic reticulum. *Curr. Opin. Cell Biol.* **16**, 343–349
- Swanton, E., High, S. and Woodman, P. (2003) Role of calnexin in the glycan-independent quality control of proteolipid protein. *EMBO J.* **22**, 2948–2958
- Spampanato, J., Kearney, J. A., de Haan, G., McEwen, D. P., Escayg, A., Aradi, I., MacDonald, B. T., Levin, S. I., Soltesz, I., Benna, P. et al. (2004) A novel epilepsy mutation in the sodium channel SCN1A identifies a cytoplasmic domain for beta subunit interaction. *J. Neurosci.* **24**, 10022–10034
- Miller, E. A., Beilharz, T. H., Malkus, P. N., Lee, M. C., Hamamoto, S., Orci, L. and Schekman, R. (2003) Multiple cargo binding sites on the COPII subunit Sec24p ensure capture of diverse membrane proteins into transport vesicles. *Cell (Cambridge, Mass.)* **114**, 497–509
- Wallace, R. H., Wang, D. W., Singh, R., Scheffer, I. E., George, Jr, A. L., Phillips, H. A., Saar, K., Reis, A., Johnson, E. W., Sutherland, G. R. et al. (1998) Febrile seizures and generalized epilepsy associated with a mutation in the Na⁺-channel beta1 subunit gene SCN1B. *Nat. Genet.* **19**, 366–370
- Meadows, L. S., Malhotra, J., Loukas, A., Thyagarajan, V., Kazen-Gillespie, K. A., Koopman, M. C., Krieger, S., Isom, L. L. and Ragsdale, D. S. (2002) Functional and biochemical analysis of a sodium channel beta1 subunit mutation responsible for generalized epilepsy with febrile seizures plus type 1. *J. Neurosci.* **22**, 10699–10709
- Makita, N., Bennett, P. B. and George, Jr, A. L. (1996) Molecular determinants of beta 1 subunit-induced gating modulation in voltage-dependent Na⁺ channels. *J. Neurosci.* **16**, 7117–7127
- Qu, Y., Rogers, J. C., Chen, S. F., McCormick, K. A., Scheuer, T. and Catterall, W. A. (1999) Functional roles of the extracellular segments of the sodium channel alpha subunit in voltage-dependent gating and modulation by beta1 subunits. *J. Biol. Chem.* **274**, 32647–32654

Received 30 March 2005/3 August 2005; accepted 4 August 2005

Published as BJ Immediate Publication 4 August 2005, doi:10.1042/BJ20050518