Biochimica et Biophysica Acta 1848 (2015) 1545–1551

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Benchmarking the stability of human detergent-solubilised voltage-gated sodium channels for structural studies using eel as a reference

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ARTICLE INFO

Article history:
Received 21 December 2014
Received in revised form 22 March 2015
Accepted 23 March 2015
Available online 30 March 2015

Keywords:
Human voltage-gated sodium channel
Rat VGSC
Electrophorus electricus
Membrane protein stability
Structural biology
Radioligand-binding assay

ABSTRACT

With the ultimate goal of detailed structural analysis of mammalian and particularly human voltage-gated sodium channels (VGSCs), we have investigated the relative stability of human and rat VGSCs and compared them with electric eel VGSC. We found that NaV1.3 from rat was the most stable after detergent solubilisation. The order of stability was rNaV1.3 > hNaV1.2 > hNaV1.1 > hNaV1.6 > hNaV1.3 > hNaV1.4. However, a comparison with the VGSC from *Electrophorus electricus*, which is most similar to NaV1.4, shows that the eel VGSC is considerably more stable in detergent than the human VGSCs examined. We conclude that current methods of structural analysis, such as single particle electron cryomicroscopy (cryoEM), may be most usefully targeted to eel VGSC or rNaV1.3, but that structural analysis on the full spectrum of VGSCs, by methods that require greater stability such as crystallisation and X-ray crystallography, will require further stabilisation of the channel.

1. Introduction

Voltage-gated sodium channels (VGSCs) are dynamic membrane proteins [1] that are selectively permeable to Na^+ ions when the channels are open. They are crucial for the generation and propagation of action potentials in electrically excitable tissues like muscle, heart, and nerve. Mutations of VGSCs cause multiple inherited diseases known as channelopathies [2,3], including periodic paralysis, cardiac arrhythmia, epilepsy, and neuropathic pain [4–6]. They are key medical targets for local anaesthetic or pain-alleviating drugs as well as being the sites of action of dangerous neurotoxins. Although the kinetics of VGSCs have been extensively characterised *via* electrophysiology [7], and there have been a number of early studies to characterise their secondary [8–11] and tertiary [12] structure, and to model aspects of their detailed three-dimensional (3D) structure [9,13,14], high-resolution structural information remains scanty. High-resolution structures will be essential to extend our understanding of the molecular mechanism of gating (activation and inactivation driven by a drop in the membrane potential) from bacterial to the full range of eukaryotic VGSCs, and for development of therapeutically useful drugs. High resolution structural studies would enable the development of a completely new class of drugs for alleviation of neuropathic pain or anaesthesia.

Because of their key role in human physiology, the VGSC family represents a very important area of research for structural biology. There are, however, many difficulties and challenges associated with structure analysis of mammalian VGSCs *in vitro*. We cannot emphasize strongly enough how difficult it has been even to reach the stage where structural analysis can be attempted on mammalian VGSC. Expression of quantities sufficient for structural work is one problem, but stability is probably the single most important barrier to progress. A membrane protein or membrane protein complex must be sufficiently stable for extraction from its native lipid membrane environment into, at least initially, a detergent-solubilised form. Purification often by a factor of several thousands is then required when expression levels or natural abundance levels are very low, as with VGSCs. Because their normal function in depolarizing excitable membranes requires only a few channels per square micron of membrane, VGSCs have been under strong evolutionary pressure to maintain expression levels at an appropriate and stably regulated low level. Studies have shown [15] that the VGSC α-subunits remain as a metabolically stable intracellular pool of intracellular subunits, whose cell surface expression in active form is regulated through co-expression and combination with β-subunits, especially β_2. Even while using the mildest detergents and with the addition of lipid or cholesterol-based stabilizing additives, there are classes of eukaryotic membrane protein that are intrinsically unstable once extracted from their natural membrane environment.

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http://dx.doi.org/10.1016/j.bbamem.2015.03.021

One of the most successful strategies has been to combine systematically a number of stabilizing mutations to obtain a modified protein that has been thermostabilised, while still retaining its native structure or at least a well-defined conformation that represents one of its natural conformations. This strategy has been termed conformational thermostabilisation [16,17] and has been outstandingly successful when applied to members of the G protein-coupled receptor (GPCR) family [18–21]. However, that approach depends on having a robust assay and expression levels that are sufficient to characterize many hundreds of mutations. We have not yet reached this stage for any human or eukaryotic VGSC.

Even once expression, purification and a degree of stabilization have been obtained, crystallization of the membrane protein is still required before X-ray analysis can be carried. There is a very clear correlation between the stability of a membrane protein in detergent solution and the likelihood of obtaining well-ordered crystals [22]. Alternatively, plunge-freezing is required to allow single particle electron cryomicroscopy [23] to be carried out, and this needs the creation of a thin film with a much larger air–water interface than in bulk solution. The conditions required for crystallization or plunge-freezing often do not coincide with the conditions for greatest stability. For example, addition of exogenous lipids often stabilizes the activity of a membrane protein or channel, but this increases the micelle size and makes crystallization or production of a monodisperse particle, e.g. for electron cryomicroscopy, more difficult. Whatever strategy is being used for stabilization, it is a prerequisite to start off with the most stable representative family member. This is the main purpose of the work we report here.

Nine mammalian isoforms (α-subunits, NaV1.1–NaV1.9) of VGSCs have been identified in the human genome encoded by distinct genes (*SCN1A-SCN9A*), but no high-resolution structural analysis has been obtained. Moreover, no expression or purification protocol, which would be suitable for structural studies of human VGSCs has been reported so far, so the possibility of progress on the structure determination of human VGSCs still appears to be some way off. Since VGSCs show strong sequence conservation across species and tissue-specific types [24], much of the early biochemistry on VGSCs, including the determination of the gene sequence [25], has focused on channels from *Electrophorus electricus* electroplax, where these channels constitute approximately 1% of the membrane protein. Channels from *E. electricus* are 59% identical with that of VGSCs from human muscle (hNaV1.4), 51% identical to the human heart channel (hNaV1.5), and 50% identical to the human channels located in the central and peripheral nervous systems (hNaV1.1 and hNaV1.7, respectively). The patterns of hydrophobicity and essential residues are even more closely preserved, increasing the probability that all VGSCs share very similar 3D structures [13,26].

The primary sequence shows that the sodium channel α subunit consists of four evolutionarily homologous internal repeats (domains I–IV), each of which is predicted to consist of six-transmembrane-spanning segments (S1–S6), plus linking regions of differing lengths between the domains and extended aqueous-soluble N- and C-terminal regions. Only a low resolution (19 Å) 3D map of the channel from *E. electricus* determined by single particle cryoEM has been reported [12] and this work was carried out prior to the introduction of more rigorous tests [28,29]. The map appears to show a bell-shaped molecule composed of four domains with pseudo-4-fold symmetry surrounding a central pore. However, with recent progress in validation methods [27,28], and improvements in cryoEM [30], electron detection [31] and computation [32], it should be possible to obtain a more reliable structure. Although the structure would be expected to have many of its core features in common with the known, much simpler structures of bacterial voltage-gated sodium channels [2] or eukaryotic voltage-gated potassium channels [33], the human VGSCs are more than twice the size of these proteins so there is still a great deal of missing information. Human or mammalian VGSCs are also important targets for drug development because of their central role in cardiac function (NaV1.5) and pain transmission (NaV1.7 and NaV1.8). They consist of almost 2000 residues folded into a single extensively glycosylated peptide with a total molecular mass of 260–270 kDa [26,34]. Finally the many toxin binding sites in mammalian VGSCs have no bacterial counterpart.

With the ultimate goal of detailed structural analysis of mammalian and particularly human VGSCs, we have therefore investigated the relative stability of detergent-solubilised human and rat VGSCs and compared them with electric eel VGSC.

2. Materials and methods

2.1. Materials

Electric eels were obtained from Amazon Fish Direct (UK). Membranes of Chinese hamster ovary (CHO) containing recombinant human VGSCs (hNaV1.1, hNaV1.2, hNav1.3, hNaV1.4 or hNaV1.6) were obtained from ChanTest®, USA), and Human Embryonic Kidney HEK-293 cells stably expressing rat NaV1.3 (rNaV1.3) were kindly provided by Stephen G. Waxman (Yale University, USA). Detergents: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), *n*-dodecyl-β-D-maltoside (DDM), *n*-decyl-β-D-maltoside (DM), Genapol® C-100 (genapol), and Lubrol-PX (lubrol) were obtained from Sigma-Aldrich Co., and lauryl maltose neopentyl glycol (LMNG) from Anatrace Complete protease inhibitor cocktail tablets (EDTA-free) were obtained from Roche, 3-(N-morpholino)propanesulfonic acid (MOPS) from Fisher Scientific, and 2-({2 [Bis(carboxymethyl)amino]ethyl}(carboxymethyl)amino)acetic acid (Na_2-EDTA) from VWR International. 11-^{3}H saxitoxin (11-^{3}H STX, 20 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc., and tetrodotoxin with citrate (TTX) from Tocris Bioscience or Alomone Labs. Saxitoxin (STX), batrachotoxin (BTX), brevetoxin (PbTx-2), kurtoxin (KTX), veratridine (VTD), tarantula ProTx-II (ProTx-II), and sea anemone toxin (ATX-II) were obtained from Sigma-Aldrich, and huwentoxin-IV (HuwTX) and hainantoxin-III (HaTX) from Alomone Labs Ltd. Quik-sep disposable columns, mini-disposable columns, and Zeba Micro Spin Desalting Columns 7K MWCO for ligand binding assays were from Advanced Laboratory Supplies, BioRad Laboratories Inc., and from Thermo Scientific, respectively. Sephadex G25F, and CM Sepharose were obtained from Amersham Pharmacia, and OptiPhase SuperMix from Perkin Elmer.

2.2. Isolation of electric eel membranes

Medium-sized eels (length 0.7–1.5 m) were killed by decapitation after hypothermia. The electroplax organ was dissected, cut into 2-cm slices and stored in liquid nitrogen. Thawed electroplax tissue was homogenised for 30 s with an HR1610/01 hand blender (Philips) at full speed in 4 vol. of buffer A (0.05 M MOPS pH 6.5, 5 mM Na_2EDTA, complete protease inhibitors tablets). The homogenate was strained through a filter using a cafetiere jug (Sainsbury's), and 4 layers of gauze tissue. It was centrifuged in a Ti45 rotor in an Optima™ L-100 XP Ultracentrifuge (Beckman Coulter, Inc.) at 36,000 × *g* for 30 min, at 4 °C. Pellets were pooled, homogenised using the hand blender (Philips) in 4 vol. of buffer A, and centrifuged again as before to give crude membranes. Membranes were homogenised in 0.5 vol. of buffer B (0.05 M MOPS, pH 6.5), and stored in liquid nitrogen.

2.3. Isolation of HEK-293 membranes

Suspended cells were harvested at 2500 × *g* for 5 min and then broken by passage (2–3 times) through a 0.22 mm gauge needle in buffer B. Membranes were collected by centrifugation in the TLA55 rotor of the Optima™ MAX Benchtop Ultracentrifuge (Beckman Coulter, Inc.) at 25,000 × *g* for 10 min at 4 °C.