62-Plat

Using Potassium Channels As Reporters To Deconstruct The Function And Pharmacology Of Sodium Channel Voltage Sensors

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Voltage-activated sodium (Nav) channels found in both nerve and muscle cells are crucial for the generation and propagation of nerve impulses, and as such are amongst the most widely targeted ion channels by both toxins and drugs. The four voltage sensors in Nav channels have distinct amino acid sequences, raising fundamental questions about their relative contributions to the function and pharmacological sensitivities of the channel. Dissecting these contributions, however, has been problematic because the voltage sensors are contained within pseudosubunits (domain I to IV) of a single protein. Here we show that the four S3b-S4 paddle motifs within Nav channel voltage sensors can be transplanted into four-fold symmetric voltage-activated potassium (Kv) channels and can be used as reporters to individually examine the contributions of these paddle motifs to the kinetics of voltage sensor activation and their interactions with toxins. Our results show that each of the four Nav channel paddle motifs can interact with toxins from tarantula venom (PaurTx3, ProTx-I, ProTx-II, HaTx and SGTx1) or scorpion venom (AaHII and TsVII), that multiple paddle motifs are often targeted by a single toxin, and that the profiles of toxin-paddle interactions vary for different subtypes of Nav channels. The paddle motif from domain IV is unique because it slows voltage sensor activation and toxins must selectively target this motif to alter Nav channel fast inactivation. In contrast, toxins that interact with paddle motifs in domains I-III influence Nav channel opening. The influence of domain-specific interactions has important implications for developing strategies to reshape Nav channel activity. Therefore, our reporter approach and the principles that emerge will be useful in generating new drugs for treating pain and Nav channelopathies.

63-Plat

Persistently "Leaky" Nav Channels In Traumatized Axons: Lowered Barriers To Nav1.6 Voltage Sensor Motions In Blebbed Plasma Membrane As A Possible Explanation

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Mechanical trauma of CNS nodes of Ranvier generates axolemmal blebs. The nodal Na^+-channel, Nav1.6, leaks Na^+ after traumatic brain injury but its molecular ''lesion'' is not understood. We found that traumatic stretch of Na^+-dye loaded HEK-Nav1.6 cells causes an immediate TTX-sensitive Na^+-leak. Also, using oocytes, we measured Nav1.6 current in cell-attached patches before and after pipette aspiration (which causes blebs) and observed irreversibly left-shifted g(V) and availability(V). To determine if intact cell Nav1.6 operation left-shifts with trauma, within-cell comparisons of HEK-Nav1.6 cell I_{Na} before and after traumatic stretch would be ideal, but this is impracticable. Instead, perforated patch recordings (multi-pulse protocols) of I_{Na} were obtained in a given HEK-Nav1.6 cell which was then swollen grossly (distilled water, 90-180 s) and returned to normal saline. After 5 min (for cell morphology and $[Na^+]_{ext}$ to regularize) I_{Na} was re-measured. The resulting pattern of I_{Na} changes at various voltages showed that, post-osmotrauma, both g(V) and availability(V) were left-shifted at least 5 mV and this was irreversible (10 min experiments). Time controls showed no left-shift. A simple explanation is that, post-trauma, abnormally fluid disorganized bilayer of blebbed membrane presents abnormally low energy barriers to Nav1.6 voltage sensor motions. Smaller depolarizations are thus required to elicit sensor repacking than in stiffer prebleb bilayer. To the extent that trauma-induced blebbing was non-uniform, left-shift would be ''smeared''. For mildly traumatized axons of the traumatic penumbra, such left-smeared Nav1.6 window current, by leaving no ''safe'' voltage, should prove even more excitotoxic than maximal left-shifting. Positive feedback in free-running axons would persistently elicit Na^+-leak as window currents from variously traumatized areas triggered each other. *Supported by CIHR and HSFO.*

64-Plat

Expression, Purification and Biophysical Characterization of a Superfamily of Prokaryotic Voltage-gated Sodium Channels

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Eukaryotic voltage-gated sodium channels are monomeric membrane proteins comprised of four pseudo-repeats of domains containing six transmembrane segments, and have a molecular weight of >200 KDa. Their size and complexity makes them an arduous target for production in heterologous expression systems, a necessary step in acquiring the amounts of protein needed for biophysical and structural characterization. The simplified, single domain bacterial sodium channel containing six transmembrane segments isolated from *Bacillus halodurans*, NaChBac, can be expressed in *E. coli* in yields suitable for biophysical characterization and may enable successful crystallization and 3-D structure determination. Upon purification in 0.1% DDM, NaChBac is functional and associates to form a stable homotetramer (Nurani et al. (2008) *Biochemistry* 31:8114-8121).

Seven different bacterial sodium channels with significant homology to NaChBac have been expressed in *E. coli*, purified in high-yield and characterized for secondary structure, thermal stability and drug binding. The experimental ease of obtaining a pure and homogeneous sample varies amongst the superfamily of sodium channels studied. The bacterial sodium channels are extremely thermal stable but individual members differ in their long-term stability when stored at room temperature, 4°C and −80°C, and their ability to bind the drug mibefradil. These channels also differ in their ability to form stable tetramers upon purification in different detergents. The differences and similarities found in this superfamily of sodium channels may prove valuable for determining general structural features important for specific voltage-gated sodium channel functions. The ability to express, purify and reconstitute multiple active bacterial sodium channels in membrane-mimic environments provides an arsenal of resources for elucidating structural features and identifying residues important for sustaining function in voltage-gated sodium channels. *(Supported by a grants from the BBSRC to the MPSI consortium and BAW)*

65-Plat

Chimeric bacterial-human NaV1.7 sodium channels expressed in *E. coli*

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Voltage-gated sodium channels selectively transport sodium ions across cellular membranes in response to changes in membrane potential. Prokaryotic voltage-gated sodium channels are homotetramers, each monomer containing six transmembrane helices (S1–S6), consisting of a voltage-sensing subdomain (S1–S4) and a pore-forming subdomain (S5–S6). In eukaryotes, sodium channels consist of a single polypeptide chain containing four similar domains, each with six transmembrane helices (S1–S6), which create pseudo-tetrameric channels. In humans, genetic diseases associated with the NaV1.7 sodium channel isoform include loss-of-function (i.e. channelopathy-associated indifference to pain), in addition to gain-of-function inherited painful neuropathies; hence, this channel is an important target for drug discovery.

Expression of eukaryotic membrane proteins in *E. coli* is often a difficult task, resulting in cell death, no expression of the target protein, or proteins inserted into inclusion bodies. In order to enable the expression of crucial functional regions of eukaryotic sodium channels, we have developed a method for creating chimeric proteins with the N-terminal subdomain of a prokaryotic homologue, and the C-terminal subdomain of the eukaryotic protein of interest, thereby tricking the bacterial host into expressing a protein with functional regions of interest from the eukaryote. In this study we designed, constructed, expressed, and characterised a number of sodium channel chimeras containing the voltage sensor (S1–S4) from *B. halodurans* NaChBac and the pore regions (S5–S6) from domains II and III of human NaV1.7, including the S4–S5 linkers from either the bacterial or eukaryotic protein.

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66-Plat

Block of Tetrodotoxin-sensitive, Nav1.7, and Tetrodotoxin-resistant, Nav1.8, Na^+ Channels by Ranolazine

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Evidence supports a role for the tetrodotoxin-sensitive Nav1.7 and the tetrodotoxin-resistant Nav1.8 in the pathogenesis of pain. Ranolazine, an anti-ischemic drug, has been shown to block cardiac (Nav1.5) late sodium current (I_{Na}). In this study, whole-cell patch-clamp techniques were used to determine the effects of ranolazine on human Nav1.7 (hNav1.7+β1 subunits) and rat Nav1.8 (rNav1.8) channels expressed in HEK293 and ND7-23 cells, respectively. Ranolazine reduced hNav1.7 and rNav1.8 I_{Na} with IC_{50} values of 10.3 and 21.5 μM (holding potential=-120 or -100 mV, respectively). The potency of I_{Na} block by ranolazine increased to 3.2 and 4.3 μM when 5-sec depolarizing prepulses to -70 (hNav1.7) and -40 (rNav1.8) mV were applied. Ranolazine (1-30 μM) caused a concentration-dependent hyperpolarizing shift in the voltage dependence of inactivation of both channels, suggesting preferential interaction of the drug with inactivated states of the channels. Ranolazine (30 μM) caused a use-dependent block (10-msec pulses at 1, 2 and 5 Hz) of hNav1.7 and rNav1.8 I_{Na} and significantly accelerated the onset of, and slowed the recovery from inactivation of both channels. An increase of depolarizing pulse duration from 3 to 200 msec did not affect the use-dependent block of I_{Na} by 100 μM ranolazine. Taken together, the data suggest that ranolazine blocks the open

state and may interact with the inactivated states of Nav1.7 and Nav1.8 channels. The state-and use-dependent modulation of hNav1.7 and rNav1.8 Na^+ channels by ranolazine could lead to an increased effect of the drug at high firing frequencies, as in injured neurons.

67-Plat
Insecticide Binding to Voltage-gated Sodium Channels
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DDT and the pyrethroid class of insecticides target voltage-gated sodium channels. Their binding stabilises the channel open state, inducing prolonged tail currents associated with insect paralysis ('knockdown') and death. Target-site mutations conferring resistance, while a challenge for pest control programs, have provided valuable information on the location of the elusive insecticide-binding site. Homology modelling of the housefly sodium channel and automated ligand docking studies have identified a binding site consistent with resistance-associated mutagenesis data, structure-activity relationships of insecticides and their state-dependent binding activity [O'Reilly et al (2006) Biochem. J. 396:255-263]. The putative binding site, delimited by the domain II S4-S5 linker, S5 & S6 helices and domain III S6 helix, interfaces the lipid bilayer and is therefore accessible to lipid-soluble insecticide ligands. The model is supported by recent experimental results from voltage-clamp electrophysiology studies on mutant fruitfly sodium channels [Usherwood et al (2007) FEBS Letters 581:5485-5492]. The mutation T929I, predicted to inhibit DDT binding through steric hindrance, abolishes the effects of DDT on channel activity. M918, a residue predicted to form a binding contact with pyrethroids but not DDT, decreased deltamethrin potency without effecting DDT potency when mutated. We have developed methods based on circular dichroism spectroscopy to identify ligand binding to a non-insect channel system, namely the voltage-gated sodium channel NaChBac from *Bacillus halodurans* [Nurani et al (2008) Biochemistry 31:8114-8121], which will be used as a further test of the binding of various insecticides as predicted by the model.
(Supported by grants from the BBSRC)

Symposium 3: The Biophysics of HIV

68-Symp
Nucleic Acid Chaperone Activity of Retroviral Gag and Nucleocapsid Proteins
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Retroviral reverse transcription involves multiple nucleic acid rearrangements catalyzed by the nucleocapsid protein (NC). Ensemble and single-molecule studies have been used to gain mechanistic insights into the chaperone activity of retroviral NC proteins. The Gag polyprotein also appears to be a nucleic acid chaperone protein, a property that is likely to facilitate RNA genome dimerization and tRNA primer annealing. Using point mutations, truncated constructs, and individual domains of Gag, we have investigated the role of Gag's structural domains in chaperone function. HIV Gag mediates tRNA annealing at a reduced rate relative to NC. The NC domain is essential for Gag-mediated annealing, while the matrix (MA) domain appears to inhibit Gag's chaperone activity. Interestingly, inositol phosphates (IPs), which are known to bind to basic residues within MA and facilitate Gag particle assembly *in vitro*, stimulate the chaperone activity of Gag. Stimulation by IPs was shown to depend on the presence of MA residues K30 and K32, and the maximum effect was achieved at a 1:1 Gag:IP ratio. Taken together with previous data, these results suggest that IP or membrane binding by MA results in a conformational switch that stimulates Gag's ability to facilitate annealing of the tRNA primer. This work was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

69-Symp
Assembly of Ribonucleoproteins Involved in Viral RNA Trafficking
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Oligomerization of the HIV-1 protein Rev on the Rev Response Element (RRE) regulates nuclear export of genomic viral RNA and partially spliced viral mRNAs encoding for structural proteins. Single-molecule fluorescence imaging has been used to dissect the multi-step assembly pathway of this essential ribonucleoprotein under physiologically relevant conditions, revealing dynamic intermediates and the mechanism of assembly. Assembly is initiated by binding of Rev to a high-affinity site in stem-loop IIB of the RRE and proceeds rapidly by addition of single Rev monomers, facilitated by cooperative Rev-Rev interactions on the RRE. Dwell time analysis of fluorescence trajectories recorded during individual Rev-RRE assembly reactions has revealed the microscopic rate constants for several of the Rev monomer binding and dissociation steps. The high-affinity binding of multiple Rev monomers to the RRE is achieved on a much faster time scale than reported in previous bulk kinetic studies of Rev-RRE association, indicating that oligomerization is an early step in complex assembly. In addition to Rev, a variety of cellular proteins are also required for nuclear export of the viral mRNA. Hence, the single-molecule imaging system has also been used to monitor Rev-RRE complex assembly in the presence of selected cellular cofactors.

70-Symp
Insights Into The Mechanism Of Retroviral Genome Packaging And Assembly
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In HIV-1 infected cells, newly synthesized retroviral Gag polyproteins are directed to specific cellular membranes where they assemble and bud to form immature virions. Membrane binding is mediated by Gag's matrix (MA) domain, a 132-residue polypeptide containing an N-terminal myristyl group that can adopt sequestered and exposed conformations. Membane specificity was recently shown to be regulated by phosphatidylinositol-(4,5)-bisphosphate ($PI(4,5)P_2$), a cellular factor abundant in the inner leaflet of the plasma membrane (PM). We now show that phosphoinositides, including soluble analogs of $PI(4,5)P_2$ with truncated lipids, bind HIV-1 MA and trigger myristate exposure. The phosphoinositol moiety and one of the fatty acid tails binds to a cleft on the surface of the protein. The other fatty acid chain of $PI(4,5)P_2$ and the exposed myristyl group of MA bracket a conserved basic surface patch implicated in membrane binding. Our findings indicate that $PI(4,5)P_2$ acts as both a trigger of the myristyl switch and as a membrane anchor, and suggest a structure-based mechanism for the specific targeting HIV-1 Gag to $PI(4,5)P_2$-enriched membranes. Retroviral genomes contain elements within their 5′-untranslated regions (UTRs) that regulate multiple essential functions, including splicing, nuclear export, translational activation, genome packaging, and reverse transcription, among others. A number of studies suggest that these processes may be differentially regulated by RNA conformational changes. To gain insights into the structural basis for these processes, we have initiated NMR studies of intact retroviral packaging elements, including the native, dimeric 200 nucleotide core encapsidation signal (Ψ^{CES}) of the Moloney murine leukaemia virus (MLV) and the intact, dimeric 748 nucleotide 5′-UTR of the human immunodeficiency virus Type-1 (HIV-1). Progress toward the implementation of these data as restraints for structure refinement of the dimeric MLV Ψ^{CES} will be presented.

71-Symp
Mechanistic Studies of HIV Budding
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The HIV Gag protein coordinates viral trafficking, membrane binding, assembly, cofactor packaging, budding, and maturation. Late in the infectious cycle, Gag assembles on plasma membranes and forms enveloped particles that bud through the membrane. Efficient HIV budding depends on the actions of at least two cellular proteins that bind directly to conserved elements within the C-terminal p6 region of Gag: TSG101 and ALIX. Both of these proteins normally function as part of a multi-cellular pathway termed the, ESCRT pathway (Endosomal Sorting Complex Required for Transport). In the cell, the ESCRT pathway helps to sort ubiquitylated protein cargos into vesicles that bud into late endosomal multivesicular bodies (MVB), and also helps mediate the final step of cytokinesis (called abscission). Thus, HIV and many other enveloped RNA viruses have evolved to usurp the cellular ESCRT pathway and utilize its intrinsic membrane remodeling activities to bud from cells.
Recent studies have suggested that late-acting ESCRT pathway factors, including subunits of the ESCRT-III complex and the AAA ATPase, VPS4, may constrict the neck of the budding vesicle and/or mediate membrane fission. I will review evidence suggesting that ESCRT-III subunits can assemble into ''rings'' that surround the necks of budding particles, and then describe our structural, biophysical, and biochemical studies that indicate how: 1) ESCRT-III proteins change conformation as they are deposited from the cytoplasm onto the membrane, 2) ESCRT-III proteins bind and recruit VPS4 ATPases, and 3) VPS4 complexes assemble and act on their ESCRT-III substrates. These studies, together complementary studies from other laboratories, are providing a framework for understanding the mechanics of HIV budding.