1859-Plat Fluorinated Surfactants as Chaperones for Insertion/Folding of Membrane Proteins

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The principal difficulty in experimental exploration of folding and stability of membrane proteins (MPs) is their aggregation outside of the native environment of the lipid bilayer. Recently we suggested circumventing this problem with the help of fluorinated nondetergent surfactants that act as chemical chaperones [Biochemistry 2006, 45:2629]. The ideal chaperone surfactant should satisfy the following requirements: 1) prevent aggregation of MP in solution; 2) have minimal perturbation of MP structure; 3) dissociate from MP during membrane insertion; 4) not partition into lipid bilayer. Here we compared how surfactants with hemifluorinated (HF-TAC) and totally fluorinated (F-TAC) hydrophobic chains of different length compare to this ideal. First, we demonstrate using FCS of dye-labeled F-TAC and HF-TAC that neither of them will bind lipid vesicles (LUV). Thus, unlike the detergents, fluorinated surfactants do not compromise vesicle integrity even at concentrations far exceeding their CMC. Second, we examined interaction of surfactants with two model MPs, diphtheria toxin T-domain (DTT) and annexin B12, known to insert into the bilayer at acidic pH. Site-selective labeling of DTT with fluorescent dyes indicate that the surfactants do not interact uniformly and concentrate in most hydrophobic patches. The observed reduction in the efficiency of FRET between acceptor-labeled (H)F-TACs and donor-labeled DTT upon addition of LUV indicates that the protein sheds the layer of surfactant during its bilayer insertion. CD measurements suggest that the presence of surfactants does not alter the structure of model MPs. The cooperativity of the thermal unfolding transition, however, is reduced by the presence of surfactants, especially above the CMC. The linear dependence of the enthalpy of unfolding of DTT on the surfactant concentration is encouraging for future application of (H)F-TACs to determining the stability of the membrane-competent conformations of other MPs.

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1860-Plat Influence of the Hydrophobic Segment of Diacylglycerol Kinase Epsilon on the Membrane Topology and Activity of the Enzyme

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Diacylglycerol kinase epsilon (DGKε) is unique among mammalian DGK isoforms in having a segment of hydrophobic amino acids. This hydrophobic segment is predicted by simple algorithms to be a transmembrane helix. However, previous studies showed that the N-terminus of DGKε is located on the cytoplasmic side of the plasma membrane, the same side that the remainder of the protein is located. Thus, the hydrophobic segment, comprising residues 20–40, is not a transmembrane helix. Further theoretical calculations using angular dynamics showed that the Pro residue within the hydrophobic segment favors a bent conformation and that its replacement by Ala causes the segment to become a transmembrane helix. We made a P32A mutation of DGKε having a FLAG tag at the N-terminus. To compare the membrane topology of DGKε with the P32A mutant, NIH 3T3 cells have been transfected with FLAG-DGKε and its P32A vectors. Comparison of permeabilized and non-permeabilized cells, by immunofluorescence microscopy using anti-FLAG primary antibodies showed that the N-terminus of the P32A mutant is exposed on the external side of the membrane, in contrast to the N-terminus of full-length FLAG-DGKε that is exposed to the antibody only in permeabilized cells. To produce more of the proteins they were expressed in COS-7 cells. Activity assay *in vitro* showed that this mutated form of DGKε had a lower K_m and a lower catalytic rate constant for 1-stearoyl-2-arachidonoyl-sn-glycerol, compared with the wild-type enzyme, but retained its specificity for substrates with polyunsaturated acyl chains. Our studies demonstrate that DGKε is a monotopic protein that can be converted into a bitopic protein by a single amino acid replacement. This mutation also has significant consequences on the catalytic properties of the protein.

Platform AU: Voltage-Gated Na Channels

1861-Plat Intracellular Ca^{2+} Can Directly Regulate $Na_V1.4$ Through An Efl Motif In C-terminus Of The Channel

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Intracellular Ca^{2+} regulates gating of both voltage-dependent cardiac sodium channels ($Na_V1.5$) and skeletal muscle sodium channels ($Na_V1.4$) through EF- hand like (EFL) and IQ motifs in C-terminus (CT). The role of upstream EFL motif in Ca^{2+} regulation of wild type $Na_V1.4$ current is uncertain. Increasing intracellular Ca^{2+} shifts the steady-state availability of $Na_V1.4$ in the depolarizing direction compared to the Ca^{2+}-free condition. However mutations in the EFL key residues with an intact IQ motif abolishes Ca^{2+} mediated channel regulation. We conclude that the Ca^{2+} binding to the EFL motif has a critical role in controlling Na current availability in skeletal muscle through direct binding of Ca^{2+}. Since both EFL and IQ motifs are associated with regulation of fast inactivation, we evaluate the role of EFL and IQ in the myotonia-associated mutant $Na_V1.4$-F1705I, which alters fast inactivation.

1862-Plat Relative Comparison of the Effective Gating Charge between Voltage Gated K- and Na-Channels

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Previous studies have estimated the effective gating charge of K-channels to be around 13.6 elementary charges (Aggarwal and MacKinnon, 1996) and of Na-channels to be at least 12 elementary charges (Hirschberg et al., 1995). Here, we present a method to compare the effective gating charge of K- and Na-channels by means of fluorescence intensity and voltage clamp measurements.

eGFP marked voltage gated K-channels (Shaker, W434F, non-conducting) and Na-channels (Nav1.2) were expressed in Xenopus oocytes. Due to the homotetrameric structure of the K-channel, the insertion of the coding sequence of one eGFP molecule results in four eGFP molecules per expressed channel, while in the Na-channel only one eGFP molecule is expressed. Channel sequences coding for up to four eGFP molecules per channel were made, resulting in expressed K-channels with four, eight or twelve and Na-channels with one, two, three or four eGFP molecules per channel. A method using flourescence intensity was developed to obtain a relative measure of the number of channels expressed in the oocyte.

Correlation of the fluorescence intensity measurements with the total gating charge obtained by voltage clamp showed a linear dependence. Furthermore, the slope of the correlations as a function of the number of eGFP molecules per channel construct was again linear, both for K- and Na-channels. This method allows a relative comparison of the effective gating charge between K- and Na-channels.

1863-Plat Unfolding and Folding of Voltage Gated Sodium Channels from *E. electricus*

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Voltage gated sodium channels are large, dynamic, membrane proteins essential for efficient signaling in the nervous and muscular systems. Extensive pharmacological characterization has provided important information regarding the rapid conformational changes occurring during the cycle of closed, resting and activated channel states. However, little information is available regarding the folding pathway of these channels and the stability of these proteins under different environmental conditions. Using circular dichroism spectroscopy on detergent-purified voltage gated sodium channels from *Electrophorus electricus,* the loss of secondary structure accompanying chemical and thermal denaturations was quantified. The proteins appear to be quite resistant to either type of denaturation. Denatured channels do not appear to bind batrachotoxin, which has been shown to cause conformational changes detectable by circular dichroism spectroscopy in native eels proteins. Following the removal of the denaturating conditions, the extent of protein refolding was analyzed and the refolded channels were reconstituted into vesicles consisting of varying lipid composition. The activities of these sodium channels prior to unfolding and following refolding were compared.

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1864-Plat Human and Rat Nav1.8 Channel Gating and the Effects of S6 Segment Mutations

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The role of the S6 transmembrane segments in Na_V channel inactivation and local anaesthetic binding is well established for the $Na_V1.2$ subtype. However, the corresponding residues for the slowly inactivating $Na_V1.8$ subtype are yet to be investigated. Here we have studied human and rat Na_V 1.8 channels containing alanine substitutions at seven key positions within the S6 segments. The α-subunits were expressed in ND7/23 cells and currents were recorded using whole-cell patch clamp. While wildtype $hNa_V1.8$ and $rNa_V1.8$ channels displayed similar $V_{1/2}$ values for activation, appreciable differences in the $V_{1/2}$ for inactivation were observed following 15- and 4000- ms prepulses (V_h =−80 mV). For mutations N390A and V1414A of $hNa_V1.8$, and N389A, L1411A and V1415A of $rNa_V1.8$, rightward shifts in the $V_{1/2}$ for activation were observed. Following a 15-ms prepulse, the $V_{1/2}$ for inactivation was shifted to the left for I381A, N390A and Y1717A of $hNa_V1.8$, and I380A, L1411A, I1707A and Y1718A of $rNa_V1.8$. Furthermore, I380A, L1410A and Y1717A of $hNa_V1.8$, and I380A, L1411A, I1707A and Y1718A of $rNa_V1.8$ also gave leftward shifts following a 4000-ms prepulse. For $hNa_V1.8$ F1710A and $rNa_V1.8$ F1711A, the fraction of non-inactivating Na^+ currents following 30-ms depolarisations were not altered (V_h = −80 mV), in contrast to those described for the corresponding mutation in $Na_V1.2$. The lack of shifts in the $V_{1/2}$ for inactivation in V1414A and F1710A of $hNa_V1.8$ and homologous mutations of $rNa_V1.8$ contrasts with the shifts described for $Na_V1.2$. Conversely, the effect on the voltage-dependence of inactivation was greater for I381A and Y1717A of $hNa_V1.8$ and homologous mutations of $rNa_V1.8$ than described for $Na_V1.2$. Our results suggest that the S6 residues of $Na_V1.8$ influence channel gating in a distinct manner to the corresponding residues of $Na_V1.2$.

1865-Plat External Alkali Cations Impede Sodium Channel Slow Inactivation

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Voltage-gated Na channels become slow inactivated in response to sustained depolarization or to prolonged bursts of action potentials, as revealed by a slow time course of recovery lasting hundreds of msec to sec. The molecular basis of slow inactivation remains unknown, and to gain further insight into possible mechanisms we have characterized the influence alkali cations on either side of the channel. NaV1.4 channels were expressed in HEK cells and studied under whole-cell voltage clamp. Slow inactivation was markedly impeded by external Na^+ and Li^+, as manifest by a 20 mV right shift in voltage dependence. External K^+, Rb^+, or Cs^+ produced only small differences for slow inactivation compared to cation-free solution (sucrose). Varying the internal cation had no effect on slow inactivation, even when the fast-inactivation gate was disrupted. Cation specificity was not dictated by selectivity of the pore,

because a mutation (K1237C) that rendered K^+ equally permeant to Na^+ did not also cause disruption of slow inactivation by external K^+. The voltage-dependence for disruption of slow inactivation by external Na^+ was small, with an equivalent electrical distance of δ = 0.15. These data suggest occupancy of a cation binding site in the outer vestibule of the pore, external to the selectivity filter, impedes slow inactivation. The interaction appears to be allosteric, rather than a foot-in-the-door, since prior studies have show that sites within one residues of the DEKA filter are accessible to modification by MTSET in slow inactivated channels.

1866-Plat Divergent Properties of $Na_V1.1$ Mutations Associated with Familial Hemiplegic Migraine

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Familial hemiplegic migraine type-3 (FHM3) is a severe autosomal dominant migraine syndrome caused by mutations in human SCN1A, the gene encoding $Na_V1.1$. In this study we characterized the functional consequences of three mutations associated with FHM3 (L263V, Q1489K and L1649Q) in an effort to identify $Na_V1.1$ biophysical defects that underlie this form of inherited migraine. Only L263V and Q1489K generated quantifiable sodium currents when co-expressed in human tsA201 cells with the human β_1 and β_2 accessory subunits. L263V exhibited a depolarizing shift in the steady-state voltage dependence of fast and slow inactivation, accelerated recovery from fast inactivation and delayed entry into slow inactivation. In contrast, Q1489K displayed enhanced entry into slow inactivation as well as delayed recovery from fast and slow inactivation. Both L263V and Q1489K displayed a significantly increased persistent current (~ 1.5 % of peak current). The net effect of these multiple gating defects was explored using pulse train experiments. Fractional current amplitude remaining after 100 depolarizations to 0mV (5ms, holding potential -120mV, 100Hz) was significantly larger for L263V and smaller for Q1489K. The mutant L1649Q failed to generate measurable whole-cell current in our expression system. Cell surface biotinylation studies revealed that L1649Q exhibited a reduction in cell surface expression consistent with impaired trafficking, a previously unrecognized disease mechanism involving $Na_V1.1$. These data reveal that diverse $Na_V1.1$ biophysical defects can converge at the phenotypic expression of FHM3.

1867-Plat Characterizations Of *Scn5a* Mutations Associated With Sick Sinus Syndrome

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The *SCN5A* gene, encoding cardiac voltage-gated Na^+ channels (Nav1.5), is crucial to the initiation, propagation and maintenance of the cardiac rhythm. Genetic defects of *SCN5A* have been identified to associate with a variety of inherited cardiac arrhythmic disorders. We have characterized biophysical properties of eight reported sick sinus syndrome (SSS) associated *SCN5A* mutations in parallel experiments in order to establish respective genotype-phenotype relationships. Wild-type Nav1.5 and eight mutant channels (E161K, T187I, L212P, T220I, D1275N, P1298L, G1408R, W1421X) were expressed in *Xenopus* oocytes. Three mutant channels, T187I, G1408R and W1421X, did not produce any detectable current, whereas the other five mutants showed a variable degree of peak current reduction ranging from 90% to 10% compared to Nav1.5. All five variants, except E161K, displayed a significant negative shift in the voltage dependence of inactivation (between 4.2 to 15.1 mV), faster current decay but a nearly unchanged recovery from inactivation. L212P and D1275N exhibited hyperpolarizing and depolarizing shift in the voltage dependence of activation, respectively. Comparisons of our experimental results with published clinical data resulted in reasonable genotype-phenotype relationships, except for G1408R which did not generate any measurable current but was linked to a relatively slight clinical manifestation. Our present results were similar to data from transfected HEK293 cells, which suggested that results from oocytes are reliable. Inconsistencies of genotype-phenotype relationships in case of some *SCN5A* mutations imply that more factors, such as splice variants or polymorphisms, need to be considered.

1868-Plat Gating-pore Currents In $Na_V1.4$ Mutants Causing Hypokalemic Periodic Paralysis

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Hypokalemic periodic paralysis (HypoPP) is caused by missense mutations in S4 voltage-sensing domains of Na^+ ($Na_V1.4$) or Ca^{2+} (CaV1.1) channels of skeletal muscle. Recently, it was demonstrated that HypoPP mutations in the DIIS4 segment of $Na_V1.4$ expose accessory permeation pathways through aqueous crevices ("gating-pores"), separate from the central Na^+-conducting pore. There are several disparities between these reported gating-pore currents, including differences in amplitude and ionic selectivity, which are critical features in establishing their pathological relevance. To address this, we studied gating-pore conductances created by mutations at the rat $Na_V1.4$-R666 position (ortholog of the human R672 site). Substitution with glycine (R666G) created a gating-pore exhibiting permeability to both K^+ and Na^+, but not NMDG. The R666G gating-pore conductance exhibited an unusually shallow voltage-dependence at hyperpolarized voltages, and was further distinguished by robust inward rectification, even under conditions strongly favoring outward ionic current. A model incorporating a saturable, voltage-gated permeation pathway characterized by a

single cation binding site flanked by two energy barriers, asymmetrically oriented within the electrical field, is sufficient to account for these unique features. Other HypoPP mutations at R666 (S/C) had gating-pore currents with similar ionic selectivity, voltage-dependence and amplitude. In contrast, the R666H HypoPP mutation produced a gating-pore with selectivity for protons over larger monovalent cations, but exhibited similar current amplitude. The estimated inward current flowing through the gating-pores created by all R666 mutants under normal physiological conditions is small (~0.1%) relative to the current flowing through the central Na^+-conducting pore. They are thus similar in magnitude to the gating-pore proton current we previously reported in the R663H HypoPP mutant. The pathological effect of these low-amplitude currents may be to potentiate the sarcolemmal threshold for aberrant depolarization in the setting of reduced extracellular K^+.

Platform AV: Microtubules & Microtubule-Associated Proteins

1869-Plat Effects of Porphyrins on Tubulin Polymerization

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Studies suggest that irradiation of porphyrins produces direct damage of proteins including tubulin. Our previous research group's fluorescence spectroscopy investigation of the interaction of meso-tetrakis(p-sulfonatephenyl)porphyrin (TSPP) and protoporphyrin IX (PPIX) with tubulin showed that binding to the protein occurs. The binding constant for TSPP is approximately one order of magnitude higher than PPIX (3.1 ± 1 x106 M-1 vs. 2.4 ± 0.9 x105 M-1) while the number of binding sites is the same for both (~ 1). Also, the quenching of tubulin shows a larger Stern-Volmer constant for TSPP than PPIX which may indicate a different location of the binding sites. Circular dichroism of both porphyrins also showed that there is no distortion of the porphyrin macrocycle upon binding.

We also investigated the effect of the porphyrins on tubulin polymerization using turbidity assay. Polymerization studies were carried out at a 1:1 ratio of tubulin(~10μM) to porphyrin and showed that in modified MES buffer at 37°C the polymerization of tubulin proceeds very slowly except in the presence of a reagent such as taxol. These studies revealed that polymerization of tubulin occurred more slowly, increasing the nucleation phase and growth phase of microtubules. Simulation of potential porphyrin binding sites using ArgusLab showed that TSPP and PPIX have affinity to sites that do not overlap other anti-microtubule reagents such as taxol, colchicine, and vinblastine.

1870-Plat Buckling And Force Propagation Along Intracellular Microtubules

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The mechanics of most eukaryoric cells is governed by their cytoskeleton, a composite polymeric scaffold made of a variety of protein filaments and crosslinkers. Of all the cytoskeletal filaments, microtubules are the stiffest and play a crucial role in cell mechanics and intracellular transport. Recent experiments [1] have shown that the mechanical reinforcement due to the surrounding cytoskeleton allows microtubules (MTs) to bear very large compressive loads (up to 100pN), and can greatly affect force transmission along MTs. Motivated by this, we study theoretically the mechanical response of and force propagation along these stiff elastic filaments embedded in the non-linearly elastic cytoskeletal matrix. We find that, although embedded microtubules buckle when their compressive load exceeds a critical load found earlier, the resulting deformation is restricted to a finite spatial range that depends on both the non-linear material properties of the surrounding cytoskeleton, as well as the direct coupling of the microtubule to the cytoskeleton possibly through MT-associating proteins (MAPS). This gives rise to a finite, monotonic force-extension behavior.

This work shows how the range of compressive force transmission by microtubules can be as large as tens of microns and is governed by the direct coupling to the surrounding cytoskeleton.

References

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1871-Plat Numerical Modeling Exhibits The Importance Of Microtubule Bundle Formation In The Self-organized Development Of Spindle Poles

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In Xenopus meiotic extracts, despite substantial angular diffusion due to thermal effects, microtubules orient themselves to form spindle poles. This orientation appears to come primarily from the mechanical forces created by crosslinking molecular motor complexes, both plus-directed and minus-directed. Numerical simulations of a biophysical model have shed much light on this process. In our biophysical model, the plus ends of microtubules are attached by plus-directed motors fixed at chromatin surfaces. The attachment points form a random spatial distribution, with rotationally asymmetric statistics. Minus-directed crosslinking motor complexes bring the microtubules minus ends together to form the poles. Our initial hypothesis was that there was a one-stage process where individual microtubules would be captured by crosslinking motors and forced into alignment with one of the developing poles. Numerical simulations showed, however, that the assembly process involves two stages. Initially, microtubules form small bundles of two or three microtubules. These small bundles still exhibit considerable angular diffusion. Over time, these bundles coalesce into larger bundles which show smaller amounts of angular diffusion. In addition, the larger bundles react more strongly to the spherically-asymmetric distribution of chromatin attachment points. This generates a torque on large bundles that moves them toward the two pole positions. The self-similar process of bundle formation and coalescence is important to the formation of spindle poles. Actual angular alignment of microtubules with the spindle becomes important only