488-Pos
Development of Bicelles Containing Anionic Lipids to Characterize Cationic Membrane Active Peptides by NMR Spectroscopy
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Aqueous dispersions of bicelles (bilayered micelles) offer an alternative to mechanically aligned bilayer samples for solid-state NMR spectroscopy (Sanders and Landis, 1995, *Biochemistry* 43:4030-4040). Bicelle formation requires at least a binary mixture of zwitterionic long-chain and short-chain lipids. The long lipid (such as 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) forms a planar bilayer surface, while the short-chain lipid (such as 1,2-di-O-hexyl-*sn*-glycero-3-phosphocholine), caps the edges and isolates the bilayer hydrophobic core from water. When placed in a magnetic field, bicelles may spontaneously align. Because bacterial cell membranes are composed of 20%-25% negatively-charged lipids, we have sought to include an anionic lipid (such as 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol) in our bicelle preparations. To find optimal conditions, we have varied the content of the negatively charged lipid, the ratio of long to short-chain lipid (q), and the buffer used to prepare the bicelles. ^{31}P NMR spectroscopy was used to assess the alignment of the bicelles under the various conditions. Membrane active peptides, selectively deuterated at specific amino acids, will be incorporated into bicelles under optimal conditions and into mechanically aligned bilayers on glass plates. NMR spectra from membrane active peptides in mixed lipid bicelles will be compared with spectra from peptides in macroscopically oriented bilayers prepared on glass slides. Studies of cationic antimicrobial peptides, such as lactoferricin B6 (RRWQWR-NH_2) are significant because of the increased emergence of antibiotic resistant bacterial strains. It is therefore important to understand the mechanism by which such peptides exert effects on membranes which mimic the composition of bacterial cell membranes.

489-Pos
Acylated Lactoferrin Peptides Using Solid State NMR and All-Atom Molecular Dynamics Simulations
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Lactoferricin B is a cationic antimicrobial peptide with broad spectrum effectiveness. A small piece extracted from this peptide, LfB6 (RRWQWR-NH_2), has similar antimicrobial properties, which can be further enhanced by attaching a short fatty acid to the N-terminus (C6-LfB6). The exact mechanism by which antimicrobial peptides interact with bacterial cell membranes is not well understood, but it is proposed to depend on lipid composition. In contrast to mammalian membranes which are comprised primarily of neutral lipids, bacterial membranes contain a significant (~20-25%) fraction of negatively charged lipids. In the case of LfB6, the presence of two tryptophans and three arginines are thought to promote selective interaction with bacterial cell membranes. Here, we investigate the interactions of C6-LfB6 with lipid bilayers by combining solid state ^{2}H and ^{31}P NMR with an ensemble of all-atom molecular dynamics simulations running in aggregate more than 10 microseconds. In particular, we have investigated the peptides interactions with bilayers with two distinct compositions: 3:1 POPE:POPG (bacteria-like) and POPC (mammal-like). The results show that at low concentration the peptide has very little effect on the acyl chain concentration of the anionic membrane, and a more substantial effect on the zwitterionic POPC membrane. The synergy between the experimental and simulation results generates new insights into the molecular-level physics driving antimicrobial function.

Intracellular Communications & Gap Junctions

490-Pos
Functional Mapping of Connexin Pores Reveals Different Pore Topologies and the Location of a Channel Gate
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Using the substituted cysteine accessibility method (SCAM), residues in M1 in hemichannels of Cx46 and a Cx32/43 chimera, and M2 and M3 in gap junctions of Cx32 have been identified as lining the pore. We have now conducted a comprehensive comparison map of Cx50 gap junction channels and found that the orthologous residues mapped in M3 of Cx32 are also reactive in Cx50, with one exception near the extracellular end of the helix. Residues in M2 are also reactive in Cx50, but only one is in common with Cx32 (V84), while the other three sites define a different face of M2. Unlike hemichannels, no reactivity was evident in M1 of gap junction channels. We have used several strategies to minimize artifacts by reacting sites from both ends of the gap junction channel and showing that charged thiol reagents could predictably change the ion selectivity of the pore at specific sites proposed to line the pore. These studies provide the first evidence that different connexin channels have distinct topologies that can explain large and often uncorrelated differences in their channel conductance and size exclusion limits. It also suggests that the pore lining topologies of hemichannels and gap junctions may be different. We have extended these studies to map the site where the Cx32 channel closes in response to voltage. Utilizing a disease associated mutation (M34T) to induce a closed state of the channel that can be opened with voltage, we have tested accessibility of residues in M3 from both ends of the channel, and mapped the site of occlusion in the pore to one turn of the M3 helix and also detected a conformational change in M1 consistent with a rotation of this helix.

491-Pos
Gating by Voltage and Ca^{2+} in Human Connexin (cx26) Hemichannels
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Opening of connexin hemichannels permits the release of small metabolites, such as ATP and glutamate, which play an important autocrine/paracrine signaling in a variety of cell types. The recently solved crystal structure of the Cx26 gap junction channel allows us to explore in greater detail the relationship between the structure and function of both, hemichannels and gap junction channels. Here, we begin by revisiting the activation mechanisms of human Cx26 (hCx26) hemichannels by voltage and Ca^{2+}. Using the two electrode oocyte voltage-clamp technique, we found that depolarization up to +60 mV induced activation of hemichannel currents, and repolarization produced large tail-currents with slow deactivation (τ ~10 s). Interestingly, the magnitudes of the tail-currents were dependent on the lengths of the depolarizating pulses rather than the magnitudes of the currents activated during the pulses. Strikingly, cell-attached single channel recordings showed that depolarizating pulses ($\leq$40 mV) stimulated only infrequent and brief openings of hCx26 hemichannels. However, the repolarizating pulses induced opening of single hemichannel currents (likely corresponding to the tail currents) with smaller conductance and longer mean open times. In addition, we found that low Ca^{2+} (500 μM) increased macroscopic hemichannel currents at positive potentials and slowed deactivation of the tail currents. We are currently performing single channel recordings to elucidate how Ca^{2+} modulates channel activation and deactivation kinetics in response to depolarizing and repolarizing voltages. Our results indicate that while depolarization causes opening of some hCx26 hemichannels, it mostly shifts the hemichannels to a non-conductive state that will likely open after repolarization; and that Ca^{2+} ions may play a role by regulating the energetics of these transitions. Supported by The Grass Foundation.

492-Pos
Structures of Connexin26 Mutants Demonstrate a Global Flexibility of Subunits and N-Terminal Rearrangements in the Pore
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Gap junction channels are unique in that they possess multiple mechanisms for channel closure, however, structural insights into the gating mechanism have been limited by the lack of isolation of closed versus open channels for each gating mechanism. Here, we present three dimensional maps of the mutant connexin26 (Cx26M34A) and an N-terminal deletion of this mutant (Cx26M34Adel2-7) at 6 and 10 Å resolution, respectively, determined by electron crystallography. Three of the six connexin subunits in the Cx26M34A hemichannels have non-equivalent configurations resulting in a departure from strict hexagonal symmetry. The volume of the density seen in the pore of the Cx26M34A channels is prominently decreased in the Cx26M34Adel2-7 pore, but a slim density still resides. A projection map of Cx26 wild type (Cx26WT) channel at 10Å resolution and crystallized under conditions promoting a closed state reveals a density in the pore that is weaker than the Cx26M34A plug, however the high variance peak from the crystallographic

averaging indicates that the Cx26WT N-terminus is very flexible. The fitting of a recent X-ray crystallographic structure of Cx26 into the Cx26M34A and Cx26M34Adel2-7 2D crystal maps reveals radial shifts of the transmembrane helices toward outside of the channel. This movement of the six monomers within each of the two hemichannels may reflect a difference between an open state (3D crystals) and a closed state (2D crystals), but also reflects that in the 2D crystals, the channels are surrounded by two lipid bilayers. In addition, the channels in the 2D crystals show complex structural features at cytoplasmic side distinct from the 3D crystal structure. Thus, flexibility of inter subunit interactions and rearrangement of an N-terminus to form a ''plug'' create a closed channel for the M34A mutant.

493-Pos

Oligomeric State of Purified Wild-Type and Deafness-Associated Mutants Solubilized in Decylmaltoside

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Gap-junction channels are formed by head-to-head docking of two hemichannels, which are connexin hexamers. Gap-junction channels and hemichannels are permeable to large hydrophilic solutes (up to M_r ~1,000, depending on the isoform). Mutations of Cx26 are the most frequent cause of genetic deafness. In these studies, we expressed wild-type Cx26 with a C-terminal His tag to aid in affinity purification. Purified Cx26 solubilized in decylmaltoside was subjected to analytical gel filtration to determine its oligomeric state. We found that purified Cx26 consists of a number of oligomeric states, including monomers, hexamers and dodecamers. Wild-type Cx26 hexamers reconstituted in liposomes formed functional hemichannels, as demonstrated by sucrose-permeability assays. Purification in the presence of the reducing agent TCEP yielded more hexamers and less aggregates, whereas further incubation with TCEP resulted in an increased fraction of Cx26 monomers. We have shown that the dominant mutant R75W is incapable of forming gap-junction channels, but forms hemichannels with altered voltage dependence of the open probability, without changes in single-channel conductance. The R75A mutant does not form functional hemichannels. The fraction of Cx26 that is present as hexamers was similar for the wild type Cx26 and the R75W mutant, but it was reduced significantly in the case of the R75A mutant. The latter also displayed a significant increase in aggregation. These results suggest that the single R75A mutation decreases Cx26 hemichannel stability, which associates with the absence of functional hemichannel formation in frog oocytes injected with Cx26 R75A cRNA. This work was supported in part by NIH grants R01GM79629 and R21DC007150, American Heart Association Grant-in-Aid 0755002Y, and a grant from the Center for Membrane Protein Research of TTUHSC.

494-Pos

Deafness Mutation A88S Induces Cell Death Due to Impairment of the Slow Gating of HCX26 Hemichannel

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Connexin hemichannels can be gated by both extracellular Ca^{2+} and membrane voltage. The latter gating has two components, fast and slow, which transit the channels from open to subconductance or fully closed state, respectively. Here, we show that a non-syndromic sensorineural deafness mutation of human Cx26A88S, impairs the slow gating responsible for the depolarization induced opening and hyperpolarization induced closing of these hemichannels, while leaving the fast gating response of hemichannels and all gating of gap junction channels unaffected. Thus, under hyperpolarizing voltages comparable to cochlear supporting cells, Cx26A88S hemichannels have a persistent current that WT hemichannels do not have. As a consequence, expression of Cx26A88S in *Xenopus* oocytes induces a dramatic increase in cell lysis compared to those expressing WT hemichannels, an effect that is blocked by incubation in 2 mM extracellular [Ca^{2+}]. This is the first implication of a role for the slow voltage gating of hemichannels in the etiology of deafness. Intriguingly, the deafness mutation at A88 in Cx26 is actually the wild-type residue in Cx50, which forms hemichannels that show a persistent residual current in low extracellular Ca^{2+}. A Cx50S89A mutant confers depolarization activation, and a slow gating response that completely closes the channel, similar to the properties of wt Cx26. Thus, we have identified a residue critical to slow gating of hemichannels, but not fast gating or gating of gap junctions, that is conserved in the connexin family, and is important for different tissue functions. The location of this residue in the recently published crystal structure of Cx26 indicates that substitution of A88 with serine could result in a hydrogen bond with R143 on the third transmembrane segment, potentially limiting a critical movement required for slow gating of hemichannels.

495-Pos

Hemichannels in Thymocytes: Participation in Apoptotic Processes

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The body of evidence suggesting the existence of functional hemichannels has been increasing in different organs such us: heart, brain, and ear. However, the presence of functional hemichannels in immune system cells remains an open question. Previously, we demonstrated that thymocytes express Cx30.3 and Cx43. Nevertheless, they do not form functional gap junction channels between them or with thymic epithelial cells. For this reason, we decided investigated if the connexin found in thymocytes could function as hemichannel. We observed a generation of an ionic current and dye uptake when thymocytes are submitted to low extracellular calcium (permeabilization assay and flow cytometry) and a positive pipette potential [Vp (patch clamp technique)]. We demonstrated that hydrocortisone could modulate the thymocytes hemichannels open probability, even in the presence of 1 mM of extracellular calcium. Since hydrocortisone is a potent apoptotic inducer we tested if caspases could be implicated on the hemichannel open. We found that the caspase-9 blocker inhibited the hemichannels open. We showed the presence of functional hemichannel in thymocytes and we suggested that the opening of hemichannel could be involved in the thymocyte apoptosis.

496-Pos

Vascular Gap-Junction Cx37 Uncoupling By Tumor Necrosis Factor is Dependent on ZO-1 Expression

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Regulation of gap junctional intercellular communication plays a very important role in many physiological and pathophysiological processes. Despite significant knowledge of the role of endothelial cells during inflammation, the function of specific endothelial connexins during inflammation is not well understood. Our hypothesis is that tumor necrosis factor (TNF) will decrease gap junction dependent cell-to-cell communication of vascular connexin by disturbing connexin-cytoskeleton interactions.

Transformed HeLa cells expressing vascular connexin 37 (gift from Dr. Klaus Willecke) were used in these experiments. HeLa cells were treated with TNF (20 ng/ml) for up to 2 h. In dye-transfer experiments, Alexa Fluor-480 (HeLaCx37) was injected into one cell for 10 s and the number of labeled cells counted after 10 m. Cell lysates were prepared and ultracentrifuged. ZO-1, N-cadherin, actin, and Cx37 were detected by Western blot. Cx37 was also immunoprecipitated (IP) overnight and precipitated.

After 1 hour, TNF treatment resulted in near total loss of dye-coupling in HeLaCx37 ($p<0.02$, n=16) and remained constant up to 2 hours. siRNA-mediated knockdown of ZO-1 restored dye coupling. TNF caused a significant increase in detergent solubility of Cx37. ZO-1 was co-IP with Cx37 only after TNF treatment, suggesting that TNF induces a ZO-1 and Cx37 interaction. Actin was co-IP with Cx37 but TNF did not affect this association. N-cadherin was not co-IP with Cx37. Immunofluorescence double labeling for Cx37/ZO-1 and Cx37/actin confirm the co-IP experiments.

TNF reduces gap junction coupling of Cx37 when expressed alone in epithelial cells. The loss of Cx37 function maybe due to the loss of detergent resistance, suggesting dissociation of Cx37 plaque. TNF mediates Cx37 interaction with ZO-1 but not actin. N-cadherin does not interact with Cx37. TNF may affect Cx37/ZO-1 interaction resulting in reduced dye coupling.

497-Pos

Calcium-Calmodulin Regulation of Connexin43 Involves a Cytoplasmic Loop Domain

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Connexin43 (Cx43) is widely expressed throughout the mammalian body and is the predominant gap junction protein in the ventricular myocardium. Cx43 was recently reported to contain a calmodulin (CaM) binding site on its cytoplasmic loop (CL) domain near its third transmembrane domain (Zhou et al., JBC 282: 35005-17, 2007). Intracellular calcium (Ca_i)-dependent regulation of cardiac and Cx43 gap junctions has also been reported, but the function of this putative CaM-binding site has never been directly examined. In dual whole cell patch clamp experiments, murine neuro2a (N2a)-Cx43 cell gap junctional conductance (g_j) declined by 95% within 10 min (n=3) during bath perfusion with 1 μM ionomycin + 1.8 mM external Ca^{2+} (Ca_o). Cx43 g_j declined by only