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

Mariana C. Fiori, Lan Guan, Luis Reuss, Guillermo A. Altenberg.
Texas Tech University Health Sciences Center, Lubbock, TX, USA.

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

Ji Xu, Bruce J. Nicholson.
UTHSCSA, San Antonio, TX, USA.

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

Robson X. Faria[1], Paula Candida Fonseca[1], Cristiano Gonçalves Pontes[2], David C. Spray[3], Luiz Anastacio Alves[1].
[1]FIOCRUZ, Rio de Janeiro, Brazil, [2]CEFETEC, Rio de Janeiro, Brazil, [3]Albert Einstein College of Medicine, New York, NY, USA.

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

Yves Ouellette, Jenna Borkenhagen, Leonid G. Ermilov, Gary C. Sieck.
Mayo Clinic, Rochester, MN, USA.

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

Qin Xu[1], Yanyi Chen[2], Jenny J. Yang[2], Richard D. Veenstra[1].
[1]SUNY Upstate Medical University, Syracuse, NY, USA, [2]Georgia State University, Atlanta, GA, USA.

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

20% in nominally zero Ca_o (n=5). This Ca^{2+}-dependent uncoupling was demonstrated to be CaM-dependent by acute (10-15 min) pretreatment with 2 μM calmidazolium, wherein Cx43 g_j declined by < 10% within 10 min (n=4). To directly test for the involvement of the Cx43 amino acid residue #136-158 domain in this Ca^{2+}/CaM-dependent gap junction uncoupling process, 1 μM peptides were added to both whole cell patch pipettes and the 1 μM ionomycin/1.8 mM Ca_o perfusion experiments were repeated. The Cx43 #136-158 sequence mimetic peptide (K_d(CaM) = 860 nM) effectively prevented the Cx43 g_j decline (< 3%, n=4) whereas a scrambled sequence peptide control failed to prevent the Ca^{2+}-induced rundown of Cx43 g_j (<90%, n=3). These data unequivocally demonstrate that influx of external Ca^{2+} induces closure of Cx43 gap junctions in a CaM-dependent process involving the Cx43 residue #136-158 CL domain. This process has significant implications for the modulation of cardiac g_j by Ca_i and the ''healing-over'' of infarcted myocardium. Supported by NIH grants GM62999 & EY-05684 to JJY and HL-042220 to RDV.

498-Pos

Cam Interaction and Binding Mode Study with Peptide from Intracellular Loop of Cx50

Yanyi Chen[1], Yubin Zhou[1], Hing-Cheung Wong[1], Xianming Lin[2], Qin Xu[2], Monica M. Lurtz[3], Charles F. Louis[3], Richard D. Veenstra[2], **Jenny J. Yang**[1].
[1]Georgia State University, Atlanta, GA, USA, [2]Upstate Medical University, Syracuse, NY, USA, [3]University of California, Riverside, CA, USA.

Connexin 50 (Cx50) is a member of the α family of gap junction proteins expressed in the lens of the eye where it has been shown to be essential for normal lens development. We have identified calmodulin (CaM) binding sites in the intracellular loop near to the 3rd transmembrane region of Connexin43 (Cx43) and Connexin44 (Cx44) which belong to the same α connexin family as Cx50. Sequence alignment of the candidate CaM binding regions of Cx43 and Cx44, with Cx50 identified a region encompassing residues 141-166 of Cx50 with a high predicted affinity for CaM. A peptide $Cx50_{141-166}$ was synthesized to study the interaction of CaM with this domain of Cx50. Biophysical results indicate that in the presence of Ca^{2+}, $Cx50_{141-166}$ binds with high affinity (0.14 μM) to CaM, as monitored by NMR spectroscopy and measured by the fluorescence intensity change of IAEDANS that was covalently attached to the C-terminal Cys of CaM. Electrophysiological data support the hypothesis that elevated intracellular Ca^{2+} concentration inhibits Cx50 gap junctions because omission of Ca^{2+} from the 1 μM ionomycin bath saline prevented the 95% decline in junctional conductance (g_j) observed in the presence of 1.8 mM $CaCl_2$. This is likely CaM-mediated, because inclusion of a CaM inhibitor also prevented this Ca^{2+}-dependent inhibition of Cx50 gap junctions. The involvement of the Cx50 CaM binding domain in this Ca^{2+}/CaM-dependent regulation was further demonstrated by inclusion ofthe $Cx50_{141-166}$ peptide in both whole cell patch pipettes, which effectively prevented the usual decrease in Cx50 g_j. These results demonstrate that the binding of Ca^{2+} -CaM to the intracellular loop of Cx50 is critical for mediating the Ca^{2+}-dependent inhibition of Cx50 gap junctions in the lens of the eye.

499-Pos

Gating Modulation of Connexin45 Gap Junction Channels By Intracellular pH

Nicolas Palacios-Prado, Stephen Briggs, Feliksas Bukauskas.
Albert Einstein College of Medicine, Bronx, NY, USA.

Intracellular pH (pH_i) changes considerably during pathological conditions such as ischemia or epilepsy. Changes of pH_i affect the way cells communicate through gap junction (GJ) channels, and therefore disturb normal tissue function. To study pH_i-dependent modulation of GJ channels, we used HeLa cells expressing connexin45 (Cx45) or its fusion form with EGFP. The latter along with electrophysiological data allowed us to estimate the proportion of functional channels (N_F) within a junctional plaque (JP). We examined how junctional conductance (g_j) depends on pH_i and how pH_i affects voltage-gating properties of Cx45 homotypic and Cx45/Cx43-EGFP heterotypic GJs. Even at pH_i= ~8, where the probability of the channels being fully open approximates 1, g_j was maximal but only 5 % of the channels were functional. Changes in pH_i from ~7.2 to ~8 increased g_j ~1.8-fold in homotypic Cx45 GJs; g_j-pH_i dependence was sigmoidal with pK_a = ~7. We used a stochastic four-state model of contingent gating to fit experimental g_j-V_j dependence, which allowed us to define parameters characterizing voltage-gating sensitivity ($V_0$and A) and N_F. We found that alkalization increases g_j mainly by increasing V_0, i.e., voltage at which open and closed states of hemichannel are at equilibrium. On the other hand, uncoupling by acidification was due to a decrease of both V_0 and N_F. In both cases, the constant A, characterizing the steepness of g_j changes over V_j remained stable. These results agree with data obtained from heterotypic Cx45/Cx43-EGFP GJs in which pK_a= ~6.7, i.e., in between pK_as of Cx43-EGFP and Cx45 homotypic GJs. In summary, pH_i modulates V_j-gating that largely explains observed pH-dependent changes of cell-cell coupling.

500-Pos

Single Channel Connexin43 Plaque Formation

Cole Brokamp, David Wendell, Carlo Montemagno.
University of Cincinnati, Cincinnati, OH, USA.

Connexin43 (Cx43) is one of the most ubiquitous gap junction proteins in the human body and plays an essential role in cell-to-cell communication for a variety of organs and organ systems. Gap junction hemichannels are composed of six, often identical subunits, which can range from 26 kD to 60 kD, which assemble into water tight ion conduits which bridge the extracellular space between opposing cells and allow transfer of electrical impulses and small solutes up to 1 kD. Single hemichannels have been shown to remain functional in a cell membrane even when unopposed and have been linked to propagation of intercellular calcium waves, release of NAD+ and ATP, neuronal signaling, and the activation of many different kinase cascades. Here, we explore the electrophysiological properties of single Cx43 and Cx43eGFP hemichannels and their interactions during plaque formation in a planar lipid membrane (BLM). The average conductance of a Cx43 channel was found to be 753 ± 31 pS (n = 30) for a 500 mM KCl buffer. Cx43eGFP exhibited an average conductance of 783 ± 53 pS (n = 30). Unlike in-vivo patch clamp experiments, Cx43 was purified and isolated from other membrane constituents, producing a system capable of probing both connexon electrophysiology and the roles of several well known gap junction blockers, namely: lanthanum, carbenoxalone and lindane. We also use single channel BLM to examine the critical number of hemichannels required for plaque formation and the emergent electrical properties therein.

501-Pos

New Classes of Gap Junction Channel Blockers for Cx43 and Cx50

Silke B. Bodendiek[1], Clio Rubinos[2], Miduturu Srinivas[2], Heike Wulff[1].
[1]University of California, Davis, CA, USA, [2]SUNY State College of Optometry, New York, NY, USA.

In many tissues gap junction channels as well as hemichannels play important roles in intercellular electrical and biochemical coupling, cell synchronization, differentiation, growth and metabolic coordination. Therefore they have been proposed as potential new targets for the treatment of diseases such as epilepsy, cardiac arrhythmia and cancer. However, highly specific and potent pharmacological tools to further study their physiological as well as pathophysiological role are missing. The existing gap junction channel modulators are either of low potency, cross-react with other ion channels or exhibit no subtype specificity. To identify potent and selective gap junction blockers we screened a small library of compounds containing ion channel modulating pharmacophores. We identified five small molecule chemotypes including quinolines and triarylmethanes (TRAMs) that inhibited intercellular coupling via Cx43 or Cx50 in the lower micromolar range.

The triarylmethane derivatives, e.g. T66 (*N*-[(2-chlorophenyl)(diphenyl) methyl]-*N*-(1,3-thiazol-2yl)amine) (IC_{50} 3 μM), blocked Cx50 currents with IC_{50} values in the range of 1-10 μM while having only small or no effects on other gap junction channel subtypes such as Cx32, Cx36 and Cx46. The quinoline derivative SB002 (4-(4-phenoxybutoxy)quinoline) inhibited Cx50 (IC_{50} 3 μM) as well as Cx43 (IC_{50} 8.3 μM). We currently are exploring the structure-activity relationship (SAR) to increase potency and for the quinoline derivatives to shift the subtype selectivity profile towards Cx43.

We propose quinolines as well as triarylmethanes as new pharmacological tool compounds to further elucidate the physiological roles of Cx43 and Cx50 and to study their contribution to disease pathogenesis.

Supported by NIH ARRA R21NS064417.

502-Pos

Heteromultimeric Gap-Junction Channel Permeance: Directional Fluxes Simulated Using a Brownian Dynamics Model

Abhijit Mondal[1], **Alonso P. Moreno**[2].
[1]Mechanical Engineering, University of Utah, Salt Lake City, UT, USA, [2]CVRTI University of Utah, Salt Lake City, UT, USA.

Permeance for distinct connexin channels has been hard to predict. Heterotypic connexin combinations yield preferential directional fluxes of fluorescent molecules with small influence from particle charge, suggesting regulation through differences in pore's shape. We have simulated particles' movement across a 3D geometric pore's representation from X-ray crystallography following a Brownian Dynamics Model (BDM). A central prolate represents the pore's vestibule; a cone and a cylinder represents the pore's mouths for Cell 1 (C_1) and Cell 2 (C_2) respectively. Lucifer yellow molecules ($e=0$ or $-2e^-$, Stokes radius of $4.9\mathring{A}$) were represented as spheres. Particle-channel charge interactions were simulated placing a charged ring near the channel's mouth. BDM described closely particle behavior where the displacement vector (dx) was calculated using particles' diffusion matrix, net force and a random vector from