channel structure: connexin pores have constricted segments with which ABG-sugars interact, other than the size-selective filter. From the cytoplasmic side, ABG-G3 blocks Cx26 but not Cx32 or Cx26/Cx32 channels, and the wider ABG-G4 blocks with decreasing effectiveness Cx32>Cx26>Cx26/Cx32. From the extracellular side, ABG-G3 blocks Cx26/Cx32 better than Cx26, and does not block Cx32 channels, while ABG-G4 has no effect on any channels tested. If block were exclusively at the size-selective filter, the pattern of block from both sides of the pore should be identical and consistent with the PA-sugar pore sizing study. Instead, the data show that pore width varies as the selectivity filter is approached from one side or the other. Specifically, the pore lumen of homomeric Cx26 and Cx32 channels narrows on the cytoplasmic side of the selectivity filter. Intriguingly, heteromeric Cx26/Cx32 channels show unique and substantial narrowing extracellular to the selectivity filter. This is significant as most connexin channels in vivo are heteromeric, and heteromeric channels are selective amongst second messengers while the corresponding homomeric channels are not. Our data suggest a consequence of 'heteromericity' is that segment/s of a pore-lining domain are asymmetrically displaced toward the center of the lumen. Supported by GM36044, NS56509.

1454-Pos Board B298

Chemical Gating Mechanism Of Connexin26-containing Channels By Aminosulfonate

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Protonated taurine directly and reversibly inhibits homomeric and heteromeric Cx26-containing hemichannels but not homomeric Cx32 hemichannels. It is unknown if taurine interacts with Cx26 and/or Cx32 in heteromeric channels, which domains are involved, or if junctional channels are taurine-sensitive. These issues were addressed with channels composed of Cx26 and/or Cx32 with/without cleavable 3KDa carboxyl-terminal (CT) tags (T). Hemichannel activity was assessed in liposomes, and by extracellular dye-uptake in cells. In contrast to untagged hemichannels, Cx26T/Cx32 and Cx26T hemichannels were not taurine-sensitive, but Cx26/Cx32T hemichannels were. Tag cleavage (Tc, leaving 4aa at the carboxyl-terminus) restored taurine-sensitivity of Cx26Tc/Cx32 hemichannels, but taurine surprisingly narrowed rather than closed Cx26Tc hemichannels. Thus, the 3KDa CT tag blocks taurine-sensitivity, unless hemichannels also contain Cx32, and the short 4aa CT extension affects Cx26Tc channel open state. Taurine effects on junctional channels were assessed by intercellular dye-coupling. Taurine substantially reduced dye-coupling by Cx26 and Cx26/Cx32T channels, but not by Cx26T, Cx26T/Cx32 or Cx32T channels. Junctional channels therefore have identical taurine-sensitivity as their component hemichannels. An intracellular site for taurine action was shown by a membrane-impermeable blocker of taurine uptake. Thus, all data indicate taurine-induced pore closure utilizes the Cx26 CT. Taurine binding to Cx26-CT was assessed by natural-abundance 13C-HMQC-NMR. Overlapping resonances of Cx26-CT peptide in the presence and absence of taurine indicate no direct taurine binding to Cx26-CT. Peptide 'elisa' showed a pH dependent interaction occurs between Cx26-CT and the carboxyl-terminal 20aa of the Cx26 cytoplasmic loop (Cx26-CL). Acidification increases the binding affinity of Cx26-CL and Cx26-CT peptides, and only the protonated form of taurine negatively affects this interaction, suggesting that its disruption leads to channel closure. Structural analysis of Cx26-CT and Cx26-CL peptides in the presence and absence of taurine are ongoing. Supported by GM36044, DC7470.

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Quantitative Experimental Measurements And Mathematical Modeling Of Multi-cellular Dynamics In The Islet Of Langerhans

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Multi-cellular interactions and dynamics are key to mammalian physiology. Many organs such as the heart or brain have complex functionality that is brought about by the interactions between constituent cells. To discover general rules and principles that govern multi-cellular behavior, we are studying a relatively simple mammalian multi-cellular system- the islet of Langerhans. The islet is located in the pancreas and provides the sole source of the hormone insulin for regulating blood glucose levels. Thus, understanding the function of the islet is of critical importance to effectively treat diabetes. In this work, we have focused on understanding the electrical dynamics in the islet that underlie the coordinated pulsatile secretion of insulin. We have used quantitative microscopy to measure intra-cellular free calcium activity ($[Ca^{2+}]_i$) simultaneously over a large cellular population of the islet. To provide an experimental axis, we introduced graded changes in the electrical coupling between beta cells by applying both chemical inhibitors of gap junction activity as well as a genetic knockout of the gap junction protein. Upon this reduction in electrical coupling, synchronization of pulsatile electrical activity decreased throughout the islet. Furthermore, the propagating $[Ca^{2+}]_i$ waves, which serve to synchronize electrical oscillations, slowed and were disrupted as electrical coupling was reduced. Using a mathematical model of islet cell electrical activity and multi-cellular coupling, we can quantitatively reproduce this experimental data. This allows us to make quantitative predictions of the multi-cellular electrical behavior for any given level of electrical coupling, as well as expected behaviors under perturbations of other parameters, such as K^+-channel mutations. We can also hypothesize that this behavior is general for electrical coupling in other multi-cellular systems, potentially allowing us to predict the electrical dynamics in other neuro-endocrine cell systems.

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Triarylmethanes - a New Class of Connexin Blockers

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Connexins (Cx) are a family of proteins with 4 transmembrane regions, which are encoded by 21 genes in humans and which form hexameric connexons (= hemichannels). These connexons can either function as transmembrane ion channels or assemble into gap junctions that directly mediate signaling between adjacent cells by allowing the passage of ions, metabolites and signaling molecules up to 1 kd in mass. Both gap junctions and hemichannels play important roles in many tissues and have therefore been proposed as potential new targets for the treatment of epilepsy, cardiac arrhythmia and cancer. However, there are no specific and potent pharmacological tools to study the physiological as well as the pathophysiological role of connexins. The existing connexin modulators are either of low potency or cross-react with other ion channels. In order to identify potent and selective connexin blockers we screened a small library of compounds containing pharmacophores known to modulate other ion channels. From this library, we identified four new small molecule chemotypes including triarylmethanes (TRAMs) like clotrimazole and benzimidazoles like astemizole that inhibit Cx50 channels in a sub-type specific manner with IC50 values in the range of 1-10 μM while having little or no effect on those formed by Cx46, Cx36 and Cx32. We are currently exploring the structure activity relationship (SAR) of TRAMs for Cx50 inhibition and have recently identified T66 (N-[(2-chlorophenyl)(diphenyl)-methyl]-N-(1,3-thiazol-2yl)amine), which exhibits an IC50 of 3 μM. In general, the SAR of the Cx50 inhibiting TRAMs significantly differs from the SAR of KCa3.1 blocking TRAMs. We propose T66 and its derivatives as novel pharmacological tool compounds that may be used to study the physiological role of connexins.

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Pro-arrhythmic Effects Of Fibroblast-myocyte Coupling In Simulated Cardiac Tissue

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Fibroblasts comprise the majority of non-cardiac cells in normal heart and mediate the structural remodeling underlying progressive fibrosis in cardiac diseases. Recent experimental studies have shown that fibroblasts can electronically couple to myocytes via gap junctions and alter myocyte electrophysiology. However, the implications for cardiac arrhythmias are incompletely understood. In this study, we used mathematical modeling and computer simulation to investigate how fibroblast-myocyte coupling affects the dynamics of action potential duration (APD), excitation-contraction coupling, and alternans. Our major findings are: 1) Fibroblast-myocyte coupling shortens APD when fibroblast membrane conductance is high and resting membrane potential is low, but prolongs APD for other choices of conductance and resting potential. 2) Depending on the membrane conductance and resting potential of fibroblasts, fibroblast-myocyte coupling can either promote or suppress APD alternans by steepening or flattening APD restitution. 3) When alternans is calcium-driven, fibroblast-myocyte coupling always promotes alternans, and can result in electromechanically discordant alternans. 4) In cardiac tissue, fibroblast-myocyte coupling slows conduction velocity and broadens its restitution, promoting spatially discordant alternans. Spatially discordant alternans can also arise from electromechanically concordant alternans and electromechanically discordant alternans in different regions of the tissue. In conclusion, fibroblast-myocyte coupling has multiple pro-arrhythmic effects on electrophysiological properties in cardiac tissue.

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Gap Junction Permeability: Transfer of Negative and Positive Charged Probes

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Gap junction channels are composed of connexins, which exhibit specific permeability to the variety of larger solutes including second messengers, polypeptides and siRNAs.

Here, we report the permeability of solutes with different size and net charge Lucifer Yellow (443 (no counterion), -2); Carboxyfluorescein (376, -2); AlexaFluor350 (326, -1); Ethidium bromide (314, +1) and NBD-m-TMA (280, +1) through gap junction channels in HeLa cells expressing Cx26, Cx40, Cx43 and Cx45.

The channel permeability was determined using simultaneous measurements of junctional conductance and the cell-cell flux of a fluorescent probe.

All four connexins transferred negative charged probes: LY, CF and AF350, however, Cx40 and Cx26 exhibited reduced permeability when compared to Cx43. These connexins revealed following permeability ratios for LY/ CF/ AF350, respectively, relative to the ubiquitous cation K+: 0.029/ 0.032/ 0.069 for Cx43; 0.014/ 0.021/ 0.049 for Cx45; 0.0044/ 0.014/ 0.0245 for Cx26 and 0.0026/ 0.0034/ 0.0206 for Cx40.

The positive charged NBD and EthBr exhibited the following permeability relative to K+: 0.045 and 0.0125 for Cx43; 0.048 and 0.0036 for Cx45; 0.055 and 0.026 for Cx26; 0.040 and 0.009 for Cx40.

In summary, all negative charged species showed a similar permeability order: Cx43>Cx45>Cx26>Cx40. For positively charged species the permeability orders were: $Cx26 \approx Cx43 \approx Cx40 \approx Cx45$ (NBD) and $Cx26 \geq Cx43 \approx Cx40 > Cx45$ (EthBr). Reduced EthBr permeation through Cx45 channels in comparison to other connexins suggests a size-dependent discrimination of the solute. However, the reduced correlation between junctional conductance and positive charged probes flux suggests intracellular binding of the solute. Therefore quantitative comparison of positively charged solutes has to be taken cautiously.

These results confirm that channels formed from individual connexins can discriminate for solutes based on size and charge suggesting that channel selectivity may be a key factor in cell signaling.

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Stem Cell Transplantation Induces a Rapid Change in Cardiac Excitability

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Embryonic (ES) and bone marrow derived stem cells are discussed as a potential source for cardiac replacement tissue. Transplantation of undifferentiated cells into the cardiac infarct region has been shown to decrease infarct size and preserve cardiac function. Long term studies determined homing of stem cells in the cardiac muscle, the immediate impact of cell transplantation on the electrophysiological properties of however remains unclear. To determine if the time course in which stem cells establish intercellular coupling we plated Calcein/AM loaded ES cells on monolayers of cardiomyocytes (HL-1 cells). Dye transfer that was monitored by confocal microscopy, was first observed 60 min after heterocellular culture was established. It could be blocked by carbenoxolone indicating the presence of gap junction channels. After 200 min 36 ± 7% of ES cells had established intercellular coupling with cardiomyocytes. To determine the impact of cell transplantation on the electrophysiological properties we established monolayers on multielectrode arrays (MEAs). From field potential measurements we determined that induction of co-culture resulted in a biphasic change of the electrophysiological properties. During the first 45 min an increase of the conduction velocity (CV: 142 ± 17 %) and of the spontaneous beating frequency (F: 172 ± 11%) could be detected (n = 10). With further progression of co-culture however, a continuous decrease of excitability occurred (180min; F: 31 ± 3%; CV: 50 ± 2.5%) that ultimately resulted in the loss of spontaneous activity (210 min). In control cultures no biphasic change in F or CV was observed. The data indicate that stem cell transplantation results in rapid heterocellular coupling between stem cells and cardiomyocyte and a suppression of cardiac excitability. The contribution of intercellular coupling and other paracrine mechanism to the change in excitability remains to be determined.

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Posttranslational Modifications Of Connexin26 Identified By MALDI-TOF/TOF Mass Spectrometry

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Gap junctions play important roles in auditory function. Mutations in the Cx26 gene are the predominant cause of inherited nonsyndromic deafness. Some Cx26 deafness mutations directly disrupt the intercellular molecular and/or ionic signaling pathway, while others may affect the location and character of posttranslational modifications (PTMs) governing channel assembly, function and biological regulation. Mass spectrometry was used to determine if Cx26 PTMs occur at sites of deafness-causing mutations. Cx26 was isolated from HeLa cells at >95% purity by immunoaffinity followed by metal chelate chromatography using carboxyl-terminal hexahistidine and hemagglutinin tags. In-gel and in-solution enzymatic digestions were carried out in parallel with trypsin, chymotrypsin and endoproteinase-GluC. Peptides were recovered with and fractionated from reverse-phased C8 beads by stepwise elution with increasing concentrations of organic solvent. Using an ABI4800 MALDI-TOF/TOF-MS, spectra were acquired from each elution step, thereby improving detection of low abundance peptides in complex mixtures and maximizing sequence coverage. Acquisition, processing and interpretation parameters were further optimized to improve ionization and fragmentation of hydrophobic connexin peptides. MALDI-TOF-MS and MALDI-TOF-MS/MS sequence coverage values obtained were significantly above those reported for other mammalian membrane proteins. Total Cx26 sequence coverage by MALDI-TOF-MS was 75.2%, with 31.1% sequence confirmed by MALDI-TOF-MS/MS. Improved ionization and sequencing of Cx26 peptides (especially transmembrane pore-lining domains) were further achieved with a Waters nano-liquid chromatography-coupled electrospray ionization quadrupole-TOF-MS. Several different PTMs of Cx26 were identified, many of which were at sites of deafness-causing mutation. The PTMs included phosphorylation, acetylation, methylation, citrullination, hydroxylation, γ-carboxylation and palmitoylation. Knowledge of the location and character of Cx26 PTMs will be instrumental in guiding experiments to understand how cellular mechanisms of channel regulation can become altered and lead to losses in auditory function. Supported by GM36044, DC7470, NS56509 (ALH) & NS046593 (HL).

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Mutagenesis Of Charged Residues In The N-terminal α-helix Of Connexin37 Reveals An Essential Lysine Residue

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Connexins are transmembrane protein subunits that combine to form cell surface hexameric hemichannels and intercellular gap junction channels. They contain a short cytoplasmic N-terminus that has been implicated in channel gating and oligomerization. Using NMR, we previously showed that the N-terminus of human connexin37 (CX37) is α-helical between amino acids 5 and 16. The α-helical region has a hydrophilic face with three aligned negatively charged residues (E8, D12 and E16) and one positively charged residue (K9). To test their function, these charged amino acid residues were mutated individually to alanines or to other neutral amino acids. The CX37 mutants were tested for formation of gap junction plaques by fluorescence microscopy after transient transfection of HeLa cells and for formation of functional hemichannels by two-microelectrode voltage clamp after expression in single *Xenopus* oocytes. Each of the negatively charged amino acid alanine substitution (E8A, D12A, or E16A) or charge neutralization (E8Q, D12N, or E16Q) mutants formed gap junction plaques. These mutants all formed conducting hemichannels; ionic currents were of comparable magnitude to those in oocytes expressing wild-type CX37 when measured without divalent cations and were blocked by 2 mM external calcium. While gap junction plaques were observed in HeLa cells transfected with K9A, this construct did not form conducting hemichannels in *Xenopus* oocytes. No plaques were detected in a charge reversal mutant at this position (K9E). These results suggest that the negatively charged residues within the N-terminal α-helix are not individually required for formation of functional channels. In contrast, the positively charged residue, K9, is required for hemichannel opening and influences formation of gap junction plaques.

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Extracellular ATP Mediates The Intercellular Ca2+ Wave Induced By Mechanical Stimulation In Human Salivary Gland Cells

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