oocytes, whose membrane was counterstained from the extracellular side by fluorescently-labeled lectin (Alexa Fluor® 633 WGA), we show that the CNGA2 subunit significantly promotes the incorporation of the both the CNGA4 and the CNGB1b subunit within the plasma membrane of Xenopus oocytes. Activation of heterotetrameric CNGA2:A4:B1b channels was studied in excised patches by measuring simultaneously ligand binding and activation gating by using a fluorescent cGMP derivative. To study the ligand binding to the CNGA4 and CNGB1b subunit only, we performed experiments in channels with specifically disabled CNBDs. We show that cGMP activates heterotetrameric olfactory channels by binding to the CNGA2 and CNGA4 subunits only, whereas cAMP seems to activate the channels by binding to all three types of subunits. These findings suggest that the CNGB1b subunit plays an important role in distinguishing between cGMP and cAMP.

656-Pos Board B442

Functional Dimeric Organization of the Tetrameric HCN2 Pacemaker Channel

Jana Kusch[1], Susanne Thon[1], Eckhard Schulz[2], Christoph Biskup[1], Vasilica Nache[1], Thomas Zimmer[1], Reinhard Seifert[3], Frank Schwede[4], Klaus Benndorf[1].
[1]University Hospital Jena, Jena, Germany, [2]University of Applied Sciences, Schmalkalden, Germany, [3]Center of Advanced European Studies and Research, Bonn, Germany, [4]BIOLOG Life Science Institute, Bremen, Germany.

Activation of HCN pacemaker channels is exerted by hyperpolarizing voltages and further promoted by the binding of cyclic nucleotides. The channels are tetramers and the molecular processes underlying channel activation are still unknown. We studied in voltage-activated channels the binding/unbinding of a fluorescent cAMP and the induced additional activation/deactivation simultanously using confocal patch-clamp fluorometry. The obtained current and fluorescence data were globally fitted in terms of a Markovian state model containing four binding steps in both the open and closed channel plus five closed-open isomerizations. The fit provided us a set of rate constants for all transitions which allowed us in turn to estimate all equilibrium association constants, K_A, and also all equilibrium constants of the closed-open isomerizations, E. From these constants the respective Gibbs free energies (ΔG) were calculated. According to our model, the energy profile for the ligand-induced activation was considered for three distant ligand concentrations. As a result, the twofold liganded state produced an energy minimum in both the closed and the open channel. Hence, depending on the ligand concentration, states with zero, two, and four ligands are more stable than states with one or three ligands bound. This result leads us to propose that the homotetrameric HCN2 channel is functionally organized as a dimer. With respect to the equilibrium association constants, K_A, there is positive cooperativity within the two dimers and negative cooperativity between the two dimers. A consequence of this molecular organization is that ligand-induced activation is not a four-step mechanism, as suggested by an apparently fourfold symmetric tetrameric channel, but only a two-step mechanism.

657-Pos Board B443

Matrix Metalloproteinase-9 Increases the Ligand Sensitivity of CNG Channels: Evidence for Sequential Subunit Modification

Peter C. Meighan, Starla E. Meighan, Elizabeth D. Rich, R. Lane Brown, Michael D. Varnum.
Washington State University, Pullman, WA, USA.

Matrix metalloproteinases (MMPs) are a family of over 20 cell-surface and secreted, Zn^{2+}- and Ca^{2+}-dependent endopeptidases. Two closely related gelatinase MMPs, −9 and −2, have been implicated in both normal retinal processes and in several retinal diseases. MMPs are classically associated with tissue remodeling, but have been shown to influence ion channel function—either indirectly (e.g., through modification of the pericellular environment) or directly (e.g., by proteolysis of channel subunits). Because MMPs have been localized within the interphotoreceptor matrix, we hypothesized that MMP catalytic activity can influence the function of photoreceptor cyclic nucleotide-gated (CNG) channels. Cone and rod CNG channels were individually expressed in *Xenopus* oocytes and examined via patch-clamp recording in the inside-out configuration. Extracellular exposure to MMP-9 or −2 produced a profound increase in the efficacy of cAMP and the apparent cGMP affinity for both homomeric (CNGA3, $K_{1/2,GMP}$, mean ± S.E.M: $MMP9_{60min}$ = 0.62 μM ± 0.21; $control_{60min}$ = 12.6 μM ± 1.7) and heteromeric (CNGA3+CNGB3, $K_{1/2,GMP}$: $MMP9_{80min}$ = 2.2 μM ± 0.6; $control_{80min}$ = 16.4 μM ± 1.2) channel compositions. The gating changes required MMP-catalytic activity, exhibited complex state-dependence and were attenuated by N-glycosylation in the pore turret of CNGA subunits. Furthermore, MMP exposure resulted in decreased abundance of the full length CNGA3 subunit on immunoblots, and a concomitant increase in putative degradation products, consistent with proteolysis of core-channel subunits. Analyses of cGMP dose-response curves and current fluctuations suggest that MMPs generate channel subpopulations by sequentially modifying individual CNG channel subunits, where the apparent affinity for cGMP is governed by the total number of modified subunits (from 0 to 4). Together, these results provide evidence that MMPs can incrementally regulate the ligand sensitivity of CNG channels by proteolytic modification of CNGA subunits.

658-Pos Board B444

Alternative Splicing for *CNGA3* Controls Channel Sensitivity to Regulation by Phosphoinositides

Gucan Dai, Tshering Sherpa, Elizabeth Rich, Daylene Mills, Changhong Peng, Michael D. Varnum.
Washington State University, Pullman, WA, USA.

Precursor mRNA encoding CNGA3 subunits of cone photoreceptor CNG channels undergoes alternative splicing, generating variants differing in the N-terminal cytoplasmic region of the protein. In humans, four CNGA3 protein variants arise from alternative splicing, but the functional significance of these changes has been a persistent mystery. Because CNGA3 subunits mediate cone CNG channel regulation by phosphoinositides (PIs), we hypothesized that alternative splicing may tune channel sensitivity to PIs. Heterologous expression of the four CNGA3 variants alone or with CNGB3 subunits did not reveal significant differences in basic channel properties. However, inclusion of optional exon e3, with or without optional exon e5, produced heteromeric CNGA3+CNGB3 channels exhibiting an approximately three-fold greater shift in $K_{1/2,cGMP}$ after PIP_2 or PIP_3 compared to channels lacking the sequence encoded by exon e3. The relative abundance of exon e3-containing retinal transcripts was ~20% by qPCR. We have previously identified two structural features within CNGA3 that support PI regulation of cone CNG channels: an N-terminal and a C-terminal component; specific mutations within these regions eliminated PI sensitivity of CNGA3+CNGB3 channels. The e3 variant enhanced the component of PI modulation that depends on the C-terminal region, rather than the nearby N-terminal region, consistent with an allosteric effect on PI sensitivity. Alternative splicing of CNGA3 occurs in multiple species, although the exact variants are not conserved across CNGA3 orthologs. Optional exon e3 is unique to humans, even compared to other primates. In parallel, we found that a specific splice variant of canine *CNGA3* removes a region that is necessary for high sensitivity to PIs. Presumably, alternative splicing in *CNGA3* has evolved to regulate the interactions between the channel and membrane-bound phospholipids. Together, these results reveal an important mechanism of channel control that may have broad implications.

659-Pos Board B445

Characterization of Cyclic Nucleotide Gated Channels using Atomic Force Microscope

Luisa Maria Napolitano[1,2], **Alejandro Valbuena**[1], Sourav Maity[1], Monica Mazzolini[1,2], Vincent Torre[1,3].
[1]SISSA, Trieste, Italy, [2]CBM, Trieste, Italy, [3]IIT SISSA-Unit, Trieste, Italy.

Cyclic nucleotide-gated (CNG) channels have a key role in the conversion of sensory information, such as light and scent, into primary electrical signals. Structurally, these channels belong to the superfamily of voltage-gated ion channels and share a significant sequence identity with K^+ channels suggesting a common ancestral three-dimensional structure. However, unlike K^+ channels, to date no crystal structure of CNG channel has been solved. Therefore, we decided to analyze the structure of CNG channels with Force Spectroscopy using Atomic Force Microscopy (AFM), capable of giving structural information about proteins hard to crystallize in their native environment. We expressed the GFP-CNG channel in *Xenopus laevis* oocytes and isolated *Xenopus* plasma membrane containing channels' high density (~500/μm^2). Combining AFM with total internal reflection fluorescence (TIRF) microscopy, we were able to detect CNG single-molecule fluorescence attached to membrane patches. We have then proceeded to characterize CNG channels by measuring their mechanical properties by Force Spectroscopy. These measurements allow to extract a signature of their mechanical properties and to estimate their content of α-helices, β-sheet, hydrophobic and hydrophilic domains. These measurements do not require the purification or the crystallization of the CNG protein but provide important information on conformational changes during channel gating of CNG channels.