inhibition and drug washout, were not determined. Here, these issues are examined using Chinese hamster ovary (CHO) cells, transiently transfected with mouse HCN1 DNA, measurement of resulting currents using the whole-cell patch clamp technique, and a fast perfusion system for the application of lidocaine. We found that the onset of lidocaine action is fast (~6 seconds) with maximum inhibition occurring at approximately 20 seconds, and that its effects are partially reversible. With 200 μM lidocaine perfused directly onto the cell under study, If elicited in response to -85mV and -100mV test pulses was reduced by 18.4 ± 3.6 % (n= 6 cells) and 8.3 ± 1.0 % (n= 7 cells), respectively, with average current return of 49.1 ± 10.9 % and 73.9 ± 9.4 % to the level prior to lidocaine addition. The fact that currents did not return completely may be attributed to a phenomenon called current rundown, or to incomplete washout of lidocaine. The significant difference in current reduction between -85mV and -100mV suggests that the effect of lidocaine on HCN1 channels depends on voltage, with greater If inhibition at less negative voltages.

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Ligand Binding and Gating in HCN2 Channels

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HCN channels are nonselective tetrameric cation channels that are activated by hyperpolarizing voltages and modulated by the ligand cAMP. They generate spontaneous rhythmic activity in heart and brain. Ligand binding to the intracellular cyclic nucleotide-binding site accelerates activation kinetics, shifts the steady-state activation to more positive voltages and increases the open probability. Though it is relatively simple to determine an apparent affinity for the ligand action, it is not so simple to determine the true ligand affinity during channel activation because, according to the principle of reciprocity, ligand binding and efficacy depend on each other, i.e. the affinity of the binding sites must increase when the channel opens. It is therefore important to determine the binding of the ligands to the channels and channel activation simultaneously.

Activation of homotetrameric HCN2 channels was studied in inside-out macropatches simultaneously with ligand binding by means of patch-clamp fluorometry, using a fluorescent cAMP (fcAMP) that activates the channels in a similar manner as cAMP. With 1 μM fcAMP the binding of the ligand to the open channels exceeded that to closed channels. The slowness of the activation time course of HCN2 channels allowed us to monitor the ligand binding during the activation process. As predicted, the slow activation time course was accompanied by an increase of ligand binding. Moreover, the increase of binding was exponential whereas activation obeyed the typical sigmoidal time course. Hence, in the superimposed normalized time courses, the initial binding preceded activation whereas at later times activation preceeded binding. These results show that activation gating indeed increases the binding affinity for the ligands, quite as predicted by the principle of reciprocity, and that the gating of the first of the four subunits, which does not lead to channel opening, is associated with ligand binding.

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Electrophysiological Evaluation of Novel Blockers of If Current

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In the sino-atrial node (SAN) a major role in rhythm generation is played by f-channels. They mediate a sodium-potassium inward current (If) activated upon hyperpolarization and encoded by HCN genes (HCN1-4). If is overexpressed in cardiac diseases at ventricular level, where it may contribute to the increased propensity for arrhythmias. Selective f-channel blockers have a potential therapeutic use as bradycardic and antiarrhythmic agents. Zatebradine and ivabradine act as f-channel blockers but they lack cardiac selectivity, blocking the neuronal HCN isoforms. As a consequence an unmet need exists to develop new blockers selective for the mammalian SAN channel isoform, HCN4. Zatebradine analogues (C1-C5) were synthesized and the effect on If was measured on patch-clamped HEK293 cells expressing mHCN1, mHCN2 and hHCN4 and native guinea-pig and rabbit SAN cells. At 10 μM concentration all compounds reduced maximal If amplitude; however, potencies (defined by EC50) differed considerably. Ivabradine, taken as reference compound, showed no isoform selectivity; C1 and C4 were more potent on HCN1, the ratio being 4.3 (HCN2/HCN1) and 7.5 (HCN4/HCN1). C2 was more potent on HCN4, the ratio being 6 and 17 vs. HCN1 and HCN2, respectively. C3 was equipotent on HCN1 and HCN4 and C5 had low activity on all isoforms. Blockade was concentration-dependent, did not reverse upon drug removal and did not change current activation properties. Finally, data obtained in SAN cells show that effects on native If resemble those obtained on HCN4 isoform, in accordance with the hypothesis that HCN4 has a major contribution in SAN cells. Present results indicate that drug interaction with different HCN isoforms has diverse structural requirements. Current investigations are aimed to characterize the pharmacological profile of the new f-channel blockers and improve their isoform selectivity (Supported by EU - LSH M/CT/2006/018676, Normacor).

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Functional Consequences Of Disease-associated Mutations In The Pore Region Of Human Cone Photoreceptor CNG Channels

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CNGA3 encodes the A-subunit of the cone photoreceptor cyclic nucleotide-gated (CNG) channel. Mutations in the *CNGA3* gene have been associated with achromatopsia, a congenital, autosomal-recessively inherited retinal disorder characterized by lack of color vision, severely reduced visual acuity, photophobia and nystagmus. The aim of this study was the functional characterization of five mutant CNGA3 channels with amino acid substitutions in the pore region (S341P, L363P, G367V, P372S and E376K), which had been identified in achromatopsia patients. Mutant channels were heterologously expressed in HEK293 cells and their functional properties were assessed by calcium imaging and patch-clamp measurements. For patch-clamp recordings mutant CNGA3 was co-expressed with the wild-type B3 subunit present in native channels and transfected cells were incubated at 27°C in order to enhance folding and trafficking of the channel mutants. Furthermore, immunocytochemical experiments were performed after incubation at either 27°C or 37°C to determine the extent of co-localization of mutant channels with the cell membrane.

All five pore mutations rendered the channel non-functional in calcium imaging experiments, indicating severely reduced calcium influx through the mutant channel pore. Interestingly, cGMP-induced potassium currents could be recorded from patches containing channels with the mutations S341P, G367V and E376K. Even though macroscopic currents were small compared to wild type channels, these three pore mutants have been shown to possess residual potassium conductivity. While channels with the mutation G367V, P372S or E376K showed normal co-localization with the plasma membrane after incubation at 37°C, reduced surface expression was observed for channel mutants S341P and L363P, suggesting impaired folding and/or trafficking of the mutant proteins.

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Bimodal Agonism In A Cyclic Nucleotide-Gated Channel Is Coordinated By Two Adjacent Binding Domains

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Bimodal agonism is a cGMP-dependent desensitization found in the catfish CNGA2 subtype of the cyclic nucleotide-gated channel. In bimodal agonism, initial cGMP binding events at lower concentrations (<3 mM) increase channel open probability (P_o); however, additional cGMP binding events at higher concentrations (>3 mM) decrease P_o. Understanding bimodal agonism could lead to building cyclic nucleotide analogues that can specifically open or close the channel. The C-terminal region (β7-C helix) of the ligand-binding domain (BD) was previously shown to determine ligand selectivity and efficacy, so we tested its role in bimodal agonism. The corresponding region from the normal (non-bimodal) CNGA4 BD was substituted into the bimodal CNGA2 BD to form a chimeric channel subunit. We expressed the chimera as homomers and tested its activation in the excised patch-clamp. Steady-state currents were measured at high and low concentrations of cGMP. The chimera is bimodal ($I_{10mMcGMP}/I_{3mMcGMP}$ is 0.72) suggesting that the C-terminal region of the BD is not essential in bimodal agonism. The roles of intersubunit interactions in bimodal agonism were studied through fusing together two bimodal and two normal pseudo-subunits into tandem tetramers. Tetramers with the two bimodal pseudo-subunits arranged either adjacent (*cis*) or diagonally opposite (*trans*) to each other were tested. The *cis* tetramer is bimodal ($I_{30mMcGMP}/I_{3mMcGMP}$ is 0.87) but the *trans* tetramer is not ($I_{30mMcGMP}/I_{3mMcGMP}$ is 1.05). The *cis* and *trans* tetramers have an identical number of bimodal subunits yet their capability for bimodal agonism (and by extension their P_o at high cGMP concentration) is different. This suggests that the bimodal subunits could be coupled in a dimer within a ''dimer-of-dimers'' mechanism. This also suggests a putative additional cGMP binding pocket may be located in the regions between two adjacent bimodal BDs.

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The Voltage Sensor Of Cnga1 Channels Becomes Functional When The Hydrophobic Bond Between Phe380 And Leu356 Is Impaired

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The super-family of voltage gated ionic channels comprises usual voltage-dependent Na^+, K^+ and Ca^{2+} channels, but also cyclic nucleotide gated (CNG) channels which are not voltage dependent. Voltage dependency in this superfamily of ion channels is caused by the motion of the voltage sensor, identified as a positively charged transmembrane helix, referred to as S4. This voltage sensor is present in all voltage gated Na^+, K^+ and Ca^{2+} channels as well as in CNG channels, and why CNG channels are not voltage dependent although they have a voltage sensor is still an unresolved question. When Phe380, Glu363, Thr355 and Leu356 of the CNGA1 channel from bovine rods are replaced by alanine, mutant channels desensitize and exhibit significant voltage dependence. The mutant channel F380A has a reduced degree of desensitization but a very high degree of voltage dependence. Double mutant channels L356D+F380K and L356C+F380C do not desensitize, but exhibit a voltage dependent gating very similar to what observed in usual voltage gated Na^+, K^+ and Ca^{2+} channels. Therefore, when the hydrophobic bond coupling Phe380 in the upper portion of S6 to Leu356 in the P helix is impaired CNGA1 channels become voltage dependent. It is concluded that the voltage sensor in CNG channels is functional but it is inactivated by the tight hydrophobic coupling between the P-helix and S6, necessary to make the channel to open upon binding to cyclic nucleotide and not to voltage.

2465-Pos Board B435

Identification And Analysis Of CNGA3 And CNGB3 From Zebrafish

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Cyclic nucleotide-gated (CNG) channels are a crucial component of the phototransduction cascade in vertebrate photoreceptors. The opening and closure of these channels and consequently the influx of sodium and calcium ions into the photoreceptor outer segment is directed by the intracellular light-dependent cGMP level. Cone CNG channels are heterooligomers consisting of two A3- and two B3-subunits, which are encoded by the CNGA3 and the CNGB3 gene. In both genes mutations have been identified, which can lead to a dysfunction of the CNG channels in cone photoreceptors. In humans this results in the autosomal-recessively inherited disease achromatopsia (color blindness).
In order to characterize CNG channels in zebrafish, which possess four morphologically and physiologically distinct classes of cones, we have identified two homologous candidate genes for CNGA3 and two for CNGB3 by in silico database analyses. All four genes as well as a splice variant of CNGA3-1 have been cloned and were heterologously expressed in HEK293 cells. Subsequently, the zebrafish CNG channels were functionally characterized by calcium imaging and patch-clamp measurements.
The retinal expression of all four genes has been confirmed by RT-PCR. In silico analyses revealed, that the two CNGA3 candidates are located at two different locations in the zebrafish genome and are presumably a result of the whole genome duplication as it is known for several genes in zebrafish. In contrast to that, the two CNGB3 candidates are located in a tandem as a result of an additional gene duplication event. ZfCNGA3-1 and zfCNGA3-2 have 62 % identity with the human CNGA3 protein. ZfCNGB3-1 has 43 % and zfCNGB3-2 has 49 % identity with human CNGB3.

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The Cloning And Characterization Of Two Urochordate Hyperpolarization-activated Cyclic Nucleotide-modulated (HCN) Channels

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We have cloned two novel HCN genes from the urochordate species, *Ciona Intestinalis*, referred to as ciHCNa and ciHCNb, which share ~50% identity with mammalian HCN isoforms between S1 and the end of the CNBD. Based on our previous phyogenetic analysis of primary sequence, ciHCNb is more closely related to the vertebrate isoforms except that it lacks a putative N-glycosylation site near the pore, which is found in vertebrate sequences and ciHCNa. When expressed in *Xenopus* oocytes, both clones produce a slowly-activating current (I_h) in response to hyperpolarizing pulses, which is inhibited by Cs+ and ZD7288. For ciHCNb, I_h has a reversal potential of -32.4mV in 5mM K^+/91 mM Na^+ extracellular solution, which shifts to -1.9mV in 96mM K^+ solution. This suggests that I_h is carried by both Na+ and K+, a characteristic of other known HCN channels. Fitting I_h traces, generated from a -70 mV pulse, with a single exponential function yielded values for τ of 1.15 +/- 0.06s and 1.14 +/- 0.17s for ciHCNa and ciHCNb, respectively. Application of 10mM 8-bromo cAMP in the 96mM K^+ bath solution produced positive shifts in the I_h activation curve for each clone. Boltzmann fits of normalized tail current amplitudes versus test voltage (I_h activation curves) yielded $V_{1/2}$ values in the presence of cAMP of -59.86 +/- 1.62mV and -48.5 +/- 3.70mV for ciHCNa and ciHCNb, respectively. ciHCNb also produces a very large instantaneous current, which is blocked by Cs+ and ZD7288 and proportional in size to I_h. This component more similar in size to that of the sea urchin SPIH channel than to those of the mammalian HCNs. Together, the data suggest that the mammalian HCNs are evolutionarily closer to ciHCNa than to ciHCNb.

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Evolutionary Emergence of Isoform-specific Regulation by N-glycosylation in Hyperpolarization-activated Cyclic Nucleotide-modulated (HCN) Channels

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For certain ion channels in the Kv superfamily, N-glycosylation maintains stability and promotes cell surface expression; however, to our knowledge, none require it to form functional channels. To date, the role of N-glycosylation in Hyperpolarization-activated Cyclic Nucleotide modulated (HCN) channel function has been examined using only the mouse HCN2 isoform, in which, surprisingly, mutation of Asn to Gln at a predicted N-glycosylation site adjacent to the selectivity filter abolished functional expression in HEK cells. Nevertheless, other studies show that sea urchin HCN (spIH) channels are functional in HEK cells despite lacking the Asn-Xaa-Ser/Thr consensus sequon. These data raise three important questions about N-glycosylation: when in HCN evolution did it arise, do all mammalian HCN isoforms require it for function, given that they share a common ancestor with spIH, and does it affect HCN function? Here, we used phylogenetic analysis to show that invertebrates, but not chordate or urochordates, lack this N-glycosylation sequon, suggesting that it arose at a critical juncture in evolutionary time. We also show that individual mammalian HCN isoforms have distinct N-glycosylation requirements: mutation of Asn to Gln at the putative sequon renders mouse HCN2 non-functional, whereas mouse HCN1 is functionally expressed, albeit with reduced current density but minimally altered responses to voltage and cation selectivity. spIH yields robust currents but is not N-glycosylated, consistent with the absence of a predicted sequon. Taken together, these data suggest that N-glycosylation at this site emerged during chordate evolution as a regulatory mechanism that has developed uniquely for individual (uro)chordate HCN isoforms.

Neuronal Systems & Modeling

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Identifying Electrically Active Cells in Neuronal Culture and Tissue using CMOS based Multi-Transistor Arrays (MTAs)

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A unique feature of CMOS based Multi-Transistor Arrays (MTAs) compared to Metal-Electrode Arrays (MEA) is the high density of the sensor pixels over a large sensor array [1-3]. Key parameters for MTAs are a spatial resolution of 7.8µm, a temporal resolution of 6 kHz (full frame readout) and a size of $1mm^2$ (16384 sensors in total).
When using these chips for measuring the electrical activity of neurons in culture or tissue, usually the signal of one neuron is detected on several transistors. We make use of this feature to automatically identify action potentials and individual neurons in recorded data, even if the coupling area of neighboring cells overlap and therefore a sensor transistor records activity of different cells.
In a first step we detect statistically significant data by examining the combined deviation of the signal from its average on the considered transistor and its neighbors in space and time. This results in a map of data points in space and time for each action potential of all electrically active cells. By grouping signals that form cohesive neighborhoods in space and time we can identify action potentials. By examination of the cross correlation between pairs of action potentials it is possible to identify single cells, even if the coupling area of neighboring cells overlap and therefore a sensor transistor records activity of different cells. We show an application of this method to dissociated cultures of hippocampal rat neurons and rabbit retina.
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