work we present a structure for the open and closed states of the voltage-gated proton channel based upon a homology model from the Shaker channel, which has been refined with new experimental data from the labs of Peter Lars. We will also present the results of hydration studies and internal and external salt bridges in the open and closed states and what they reveal about the stabilization of the two structures.

1423-Pos Board B315
Graded Tuning of Phosphatase Activity of VSP Coupled with the Intermediate State of the Voltage Sensor
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Voltage-sensing phosphatase (VSP) consists of the voltage sensor domain and the cytoplasmic phosphatase region. We have shown that the movement of the voltage-sensor domain (VSD) is coupled to the phosphatase activity over a wide range of membrane potential (Sakata et al., 2011). However, it still remains elusive how the phosphatase activity is regulated by the movement VSD at the level of single molecule. One possible idea is that constant level of phosphatase activities is attained when VSD is in a fully activated state, and the number of molecules in the active state increases as the membrane potential becomes more positive. The other possibility is that the enzymatic activity of single VSP proteins can be graded upon distinct activated states of VSD. In this case, partial activation of VSD leads to certain level of the phosphatase activity. To distinguish between two possibilities, we studied Dr-VSP with mutations in VSD, Dr-VSP(T156R/I165R), whose Q-V plot was able to be fitted by the sum of two Boltzmann equations. Analyses of ‘gating’ current and voltage clamp fluorometry showed that VSD of this mutant moves in two steps. The enzymatic selective mutant or the protein with mutation in the linker between VSD and the phosphatase domain also showed two-step movements of the VSD. Measurements of the phosphatase activity of Dr-VSP(T156R/I165R) revealed that both transitions of VSD activation increase the phosphatase activity. These suggest that the full activation of the voltage sensor is not necessary to exhibit the phosphatase activity, and the phosphatase activity at the single protein level could be graded dependent upon the magnitude of the movement of the VSD.

1424-Pos Board B316
Correlating Residue Coevolution and Function in a Conserved Voltage-Sensing Domain
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The voltage-sensing domain (VSD) is a 4-helix transmembrane (TM) regulatory domain that undergoes a conformational change in response to a change in TM potential. While originally identified in voltage-gated cation channels, the VSD has since been observed in voltage-gated proton channels and voltage-sensing lipid phosphatases. Therefore, sensitivity to a change in TM potential appears to rely on an evolutionarily unique and structurally conserved domain. Moreover, VSDs have been fine-tuned by evolution to sense a wide range of polarization states. Here, we develop a robust hidden Markov model (HMM) of the VSD sequence for the purpose of detecting and aligning remote homologues. Then, we use statistical coupling analysis and molecular dynamics simulations to correlate highly-conserved residues and residue pairs with the atomic-level details of VSD structure, function, and tunability.

1425-Pos Board B317
Oligomeric States of Full Length Influenza a Virus M2 Proteins on Biological Membranes
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The integral matrix protein M2 of influenza a virus forms a pH-gated proton channel in the viral lipid envelope upon infecting host cells. The conductance of protons through the M2 channels in the low-pH endosomes (pH < 5.0)[1] acidifies the viral interior and facilitates the dissociation of the viral genome from the matrix protein M1. Although X-ray crystallography[2] and NMR[3] studies using truncated proteins concluded that M2 stably forms a tetrameric state of the full length M2 protein on the plasma membrane of living cells by using fluorescence resonance energy transfer (FRET) among the M2 proteins labeled with fluorophores by the coiled-coil method[4]. Contrary to previous models, M2 formed dimers at neutral pH and the dimers were converted to tetramers at pH 4.9. The tetramerization and channel activity were completely inhibited in the presence of the antiviral Amantadine hydrochloride (Am) at low pH. In contrast, the S31N mutant resistant to Am formed dimers independent of pH and the presence of Am, and the channel activity was not blocked by Am. These results indicate that the resistance of the S31N mutant could be attributed to its ability to conduct protons as dimers without forming tetramers.

References

1426-Pos Board B318
Amantadine Analogs that Inhibit Mdck Cell Infection by Influenza a with M2(S31N)
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A mutation of Ser31 to Asn has become dominant globally in human infections by influenza A since 2005, rendering the virus resistant to the FDA approved prophylactics, amantadine and rimantadine. Attempts to identify alternative M2 blockers have reportedly been futile so far. Here we report that infection of cultured Madin-Darby canine kidney cells by Influenza A/California/04/ 2009 (H1N1 swine flu), which bears the S31N mutation, is not blocked effectively by amantadine (115 uM EC50) nor rimantadine (56 uM EC50), but is effectively blocked by 11 amantadine variants. EC50s range from 1-37 uM. 8 of these 11 compounds were previously reported to block influenza A with wild type M2. As another control, an H3N2 strain of influenza A with wild type M2 (Strain Victoria from ATCC) was found to be effectively blocked by amantadine (3 uM EC50) and rimantadine, as expected. We suggest that the amantadine variants block viral reproduction by blocking the S31N strain of the M2 proton channel.

1427-Pos Board B319
Direct Observation of the Rotary Motion of FoF1-ATP Synthase Driven by Proton Motive Force
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FoF1-ATP synthase (FoF1) is a rotary motor protein which carries out ATP synthesis by coupling to the rotary motion driven by the proton motive force (pmf) across the membrane. F0,F1 consists of two molecular motors; ATP-driven motor (F1) and proton-driven motor (F0). In 1997, we for the first time observed the ATP-driven rotation of F1, and have thoroughly elucidated its operating principle as a rotary motor protein in this decade. In contrast, the proton-driven rotation of F0 or whole enzyme; FoF1, has not been observed yet. This is mainly due to the defects of pmf generation system and its interference with the detection system for rotary motions. In this year, to resolve these technical issues, we fixed these defects one by one, and eventually developed the experimental system which enabled to monitor the amplitude of pmf and the rotation of FoF1, simultaneously. In this system, we used the pH-sensitive fluorophore; pHrodo, for the measurement of proton gradient across the membrane, which is the main component of pmf. In addition, to directly observe the rotary motion of F0,F1, we attached the gold nanoparticle to the rotor part and its rotation was visualized by the total internal reflection dark-field illumination system (TIRFDF). In the presence of pmf, F0,F1 showed the clockwise rotation when viewed from F0 to F1, and accelerated its rotational rate depending on the amplitude of pmf, which was essentially consistent with the previous biochemical study. In addition to the rotational rate, we obtained various information related to the “dynamics”, such as the stepwise rotary motion coupled with the proton transport and ATP synthesis, which provide a clue for further understanding of energy conversion mechanism of F0,F1 in the physiological condition.

1428-Pos Board B320
Single Proton Pump Activity Measurements on Single Vesicles for a Quinol Heme-Copper Oxidase
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Heme-copper oxides are key elements in the respiratory chain pumping protons cellular membrane (1, 2) and generate the electrical and chemical potential that drives ATP synthesis. Understanding the precise proton pumping mechanism has been a central theme in bioenergetics research over the last decades (1, 2). However crucial mechanistic details underlying heme-copper oxidase function and regulation remain masked in conventional techniques due to ensemble averaging.
Cyclic Nucleotide-gated Channels

**1429-Pos Board B321**
Distinct Contributions of CNGA3 and CNGB3 Subunits to Ligand-Specific Activation of Cone CNG Channels
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Cyclic nucleotide-gated (CNG) ion channels regulate the electrical activity of retinal photoreceptors, rods and cones, by sensing the light-induced changes of intracellular cGMP levels. Cone CNG channels consist of CNGA3 and modulatory CNGB3 subunits, both of which contain a cyclic nucleotide-binding domain (CNBD). CNGB3 subunits confer enhanced responses to cAMP and support several aspects of channel regulation. However, it is not fully understood how CNGB3 (and CNGA3) are specialized to contribute to ligand-specific activation of cone CNG channels. Using patch-clamp recordings, we characterized several mutations located within the CNBD of CNGB3, each of which produced dramatic, ligand-specific effects on channel gating. In particular, D609M in CNGB3 reversed ligand selectivity, making cAMP a better agonist than cGMP, similar to equivalent mutations in paralogous channel subunits. These experiments suggest that mechanisms underlying ligand interaction with CNGA3 are well conserved. However, parallel mutations within the CNBD Cα-helix of CNGB3 had no effect on the ligand selectivity of heteromeric channels, consistent with the large decrement in sequence conservation in this region of CNGB3. CNGB3 appear to lack features supporting ligand discrimination. Next, we examined subunit contributions to ligand-dependent activation using CNBD “knock out” (R564E in CNGA3; R604E in CNGB3). CNGB3 R604E decreased relative cAMP efficacy, but only had a subtle effect on the cGMP activation for heteromeric channels (with wild-type or R564E CNGA3). In contrast, CNGB3 R564E caused an approximately 500-fold decrease in apparent cGMP affinity and nearly eliminated cAMP-dependent gating. Similar results were observed with analogous experiments using mutations of T565A in CNGB3 and T605A in CNGB3. Together, we propose that CNGB3 is the principal subunit mediating both ligand discrimination and ligand-dependent stabilization of the open state, while CNGB3 makes only a minor contribution to cGMP-dependent gating.

**1430-Pos Board B322**
Bacterial Roots and Branches of the HCN/CNG Family of Ion Channels: Phylogeny, Structure, and Implications for Eukaryotic HCN/CNG Structure and Function
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In gene, protein, and RNA families, it is typical for bacterial, archaeal, and eukaryotic members to form separate clusters. In this paper we report and analyze both phylogenetically and structurally a different pattern in the HCN/CNG family of ion channels, in which two subsets of bacterial members of the family clusters with eukaryotic members, rather than with the other bacterial members. The most parsimonious interpretation of the phylogeny is that this pattern is a consequence of horizontal gene transfer from a eukaryotic organism into a bacterium. We term the bacterial descendants of such transfer Eukaryotic-Like HCN/CNG’s (ELHCN/CNG’s). All of the ELHCN/CNG’s have typical potassium channel selectivity filters. Thus in that sense they are more similar to HCN’s than CNG’s. However by more global similarity measures, they are roughly equally distant from the HCN’s and CNG’s, suggesting that the eukaryote-to-bacteria horizontal transfer was of a common ancestor to both the mammalian HCN’s and CNG’s. A phylogenetic analysis further suggests that among the bacteria, subsequence of these two subsets was as much by horizontal transfer as by lineal descent. One possible mechanism for such transfer is amoeba, for which there is evidence that they engage in horizontal transfer with bacteria, and also facilitate horizontal transfer among bacteria. The ELHCN/CNG’s may be useful biophysical and functional models for eukaryotic members of this family, especially because they share with the eukaryotic members a long C-linker between the inner helix of the permeation pathway and the ligand binding sites in the cyclic nucleotide binding domain. We present a model-built structure of one of the ELHCN/CNG’s, which suggests a mechanism for coupling of ligand binding with channel opening.

**1431-Pos Board B323**
Inactivated spHCN Channel has a Decreased Binding Affinity for Camp Weihua Gao, Zhoucheng Su, Qinglian Liu, Lei Zhou.
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In response to both voltage and ligand, HCN channels play important physiological roles in the brain and the heart. HCN channels are activated by membrane hyperpolarization and the direct binding of intracellular cAMP. The spHCN channel was cloned from Sea Urchin and belongs to the HCN channel family. Different from the mammalian HCN1–4 channels, the spHCN channel exhibits strong inactivation in the absence of cAMP. Application to cAMP to WT spHCN channel abolishes the inactivation. Interestingly, a previously identified point mutation near the intracellular cAMP binding site (S6, F459L), makes the channel behave just like the mammalian HCN1 channels with any inactivation. Taking advantage of the patch-clamp fluorometry technique, we set out to investigate the dynamic, activity-dependent cAMP binding during spHCN channel gating. Surprisingly, during channel activation, we observed a decrease in cAMP binding, which is directly opposite to the observation with the HCN2 channel. Conversely, in the spHCN/F459L mutant channel, we observed an increase in cAMP binding during channel activation, which is similar to that observed in the HCN2 channel. These observations provide new insights into the intriguing communication between the voltage-dependent and ligand-dependent gating in HCN channels.

**1432-Pos Board B324**
Counting of Ion Channels on a Membrane Patch Aided by Patch-Clamp Fluorometry
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Direct estimation of the number of channels on a membrane path is important for channel biophysics. It is a classical question and has been addressed elegantly by previous studies. Especially, pioneering researchers took the advantage of fluctuations in membrane conductance caused by ion channels opening and closing. The number of channels, the single-channel current, and the probability of the channel opening can be obtained using the method of non-stationary or stationary fluctuation analysis. Here, we developed a method to count the number of channels by simultaneous electrical recording of channel opening and optical recording of the fluorescence from the green fluorescent protein (GFP) tagged to the channel. Based on the number of channels and the macroscopic current, we first tuned this method using the cyclic-nucleotide gated (CNG) channel, of which the single channel conductance and open probability are well characterized. Then we applied the I-F relationship to the hyperpolarization-activated, cAMP-regulated HCN channel, of which the estimation of single channel conductance has been controversial. We estimated that the number of channels on a piece of membrane patch could read 10,000 to 20,000 and the single channel conductance for mHCN2 channel is about 1.82 pico Siemens.

**1433-Pos Board B325**
Properties of Single HCN2 Channels Expressed in Xenopus Oocytes
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Hyperpolarization activated cyclic nucleotide-modulated (HCN) channels mediate rhythmic electrical activity in specialized brain neurons and cardiomyocytes. The channels are non-specific cation channels that are activated by hyperpolarizing voltage. Activation is enhanced by the binding of cAMP to cyclic nucleotide binding domains in each of the four subunits. In mammals four isoforms of HCN channels have been identified (HCN1–4). The single-channel conductance of HCN channels has been described first in native cardiac channels (DiFrancesco, Nature, 1986). Its value was determined to be only ~1 pS which is unusually small for a voltage gated cation channel. Surprisingly, in recombinant HCN2 channels a much larger conductance of ~35 pS was