Here we have developed a novel method to monitor for the first time with single molecule resolution the proton pumping activity of the quinol heme-copper oxidoase, cyanobacterial phytochromes I and II, and ligand-induced conformational changes of the CNG channel. Using our recently developed array of surface tethered liposomes (3-5) and coupling electrochemistry with fluorescence microscopy allowed us for the first time to simultaneously and simultaneously monitor bo.pumping activity in liposomes loaded with pH sensitive fluorescent reporters. Imaging in a massively parallel manner (10^16 liposomes) and at the single molecule enzyme level allowed to directly observe, quantify the activity rates, abundance and lifetimes, of a plethora of interconverting long-lived (min) functional states. Parallel and multiplexed single molecule readout gave access to the full distribution of rates across the ensemble of proton pumps, and importantly how pH and membrane regulatory inputs modulate the average as well as the full fluctuation spectrum of bo proton pump.

References

Cyclic Nucleotide-gated Channels

1429-Pos Board B321
Distinct Contributions of CNGA3 and CNGB3 Subunits to Ligand-Specific Activation of Cone CNG Channels
Cyclic nucleotide-gated (CNG) ion channels regulate the electrical activity of retinal photoreceptors, rods and cones, by sensing the light-induced changes of intracellular cAMP 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 regulatory 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 characterize several mutations located within the CNBD of CNGA3, each of which produced dramatic, ligand-specific effects on channel gating. In particular, D609M in CNGA3 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 Cz-helix of CNGA3 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” (RS64E 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 wildtype or R564E CNGA3). In contrast, CNGB3 R564E caused an approximately 50-fold decrease in apparent cGMP affinity and nearly eliminated cAMP-dependent gating. Similar results were observed with analogous experiments using mutations of T565A in CNGA3 and T605A in CNGB3. Together, we propose that CNGA3 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 HCN’s and CNG’s. Phylogenetic analysis further suggests that among the bacteria, subsequent spread 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, Zhioucheng Su, Qinglian Liu, Lei Zhou. VCU, Richmond, VA, USA.
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 inner activation gate in 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 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
Zhioucheng Su, Weihua Gao, Hongya Xu, Changan Xie, Qinglian Liu, Lei Zhou. Virginia Commonwealth University, Richmond, VA, USA.
Direct estimation of the number of channels on a membrane patch is important for channel biophysics. It is a classical question and has been addressed elegantly in pioneering 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 Susanne Thon, Klaus Benndorf. Institute of Physiology II, Jena, Germany.
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 mammalians 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
determined (Michels et al., Circulation, 2005). Later Dekker and Yellen (J. Gen. Physiol., 2006) confirmed a small conductance of ~1.5 pS but observed a pronounced cooperative gating of multiple channels. We expressed HCN2 channels in Xenopus oocytes and studied single channel currents in inside-out patches. Our results confirm a small conductance (~2 pS) and do not provide any evidence for a cooperative gating between the channels, enabling recording from patches containing one and only one channel. The activating effect of cAMP, applied to the bath solution, is mediated by an increase of the open probability. In conclusion, HCN2 channels expressed in Xenopus oocytes develop an only single-channel gating conductance, gate as individual channels, and are activated by cAMP via an increase of the open probability. These results are of importance for modelling single-channel properties from macroscopic currents.

1434-Pos Board B326 Voltage- and CAMP-Dependent Gating in Heterotetrameric HCN2/4-Pacemaker Channels
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HCN pacemaker channels play an important role in generating and regulating rhythmicity of special neurons and cardiac cells. They are activated by hyper-polarizing voltages and modulated by the binding of cyclic nucleotides. Four isoforms, HCN1-4, have been identified. HCN2 and HCN4, expressed in cardiac sinoatrial node and ventricular cells, build functional homotetrameric and heterotetrameric channels in various heterologous cell systems. Heterotetrameric HCN2/4 channels in Xenopus oocytes show two changes compared to each of the homotetramers: the voltage of half maximum activation is shifted to more depolarized voltages and activation kinetics are faster (Zhang et al., Biochim Biophys Acta, 2009). However, little is known about the ligand dependence of these channels. Herein, we studied the differences in activation gating of HCN2/4 channels in comparison to the respective homotetramers, thereby focusing on the effect of CAMP. We monitored activation under both steady-state and non-steady-state conditions in the presence and absence of CAMP, as well as the ligand-dependent activation kinetics after ligand jumps.

We found (1) that in HCN2/4 the apparent affinity for cAMP was between that of the two homotetramers, whereas the Hill coefficient was lowest, (2) that CAMP accelerates voltage-induced activation in HCN2/4 only slightly (factor ~2), resembling HCN4, whereas in HCN2 it accelerates activation in a voltage-dependent manner by a factor of up to ~12, (3) that the activation kinetics following a CAMP jump to channels pre-activated by voltage was fastest in HCN2/4, whereas the increase of current amplitude by a concentration jump was similar to homotetramers. Our results confirm that conformation of the HCN2 and HCN4 isoforms in Xenopus oocytes leads to heterotetrameric channels. They suggest that in native heart cells the formation of heterotetramers leads to pacemaker channels with specific characteristics, thereby fine-tuning the process of pacemaking.

1435-Pos Board B327 Voltage Gated Cation Channels Activation: Towards an Ab-Initio Kinetic Model
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HCN channels have structurally-distinct types of voltage sensors, formed by charged and hydrophobic helices. Therefore, the primary event in ligand binding has to be the rearrangement of structures of helices, thereby leading to a change in channel conformation. We propose that ligand binding causes a conformational change in the isolated CNB domain, accompanying a conformational change to an effector domain. We seek to understand the molecular details of the cyclic nucleotide activation mechanism. Our model protein is the CNB domain of a cyclic nucleotide regulated potassium channel from Mesorhizobium loti, a soil bacterium. This CNB domain is formed by a β-roll and three α-helices, namely αA, αB and αC. A major difference between the bound and unbound states is the relative position of αC helix, which raises the hypothesis that αC helix motion could be the primary event in cyclic nucleotide binding.

To address the hypothesis, we have monitored the conformational change in the isolated CNB domain. We tested two mutants having a single cysteine in αB or αC helix using a cysteine-reacting probe. Reaction kinetics were quantified by determining rate constants, which reflect the relative exposure of the cysteine. The cyclic nucleotides tested were not all equivalent in their rate constants. Moreover, the ratios between rate constants determined in the same conditions were different for αB and αC helix mutants. This indicates that ligand binding does not have the same effect on the two connected helices or, in other words, that the conformational change in αB helix is not totally dependent on that in αC helix. Therefore, the primary event in ligand binding is not the αC helix motion. Accordingly, point mutations in a functionally relevant residue in αC helix affected activity of the full-length channel only partially, suggesting that residues outside this helix must be involved in the activation mechanism.

1436-Pos Board B328 Acidic pH Uncovers Desensitization and Structurally-Distinct Types of Voltage Gating in CNGA1 Channels
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Cyclic nucleotide-gated (CNG) channels are members of the superfamily of voltage gated ion channels, but in the presence Na+ and K+ they are gated primarily by cyclic nucleotides (CNs) and only weakly by voltage. Here, we show that when extracellular pH (pHo) is decreased from pH 7.4 to 5 WT CNGA1 channels desensitize, i.e. the current activated by a steady cGMP concentration declines by 50%–80% in a voltage dependent way. Current desensitization is completely reversible upon removal of cGMP and voltage dependency of desensitization is associated to the displacement of 0.3 equivalent electronic charges across the electrical field. A very similar desensitization is observed in several mutant channels, such as E363A and T364A at the usual pH 7.4. At the desensitized state, the I/V relations are outwardly rectifying similarly in the WT CNGA1 channels at pH 5 and mutant channels E363A at pH 7.4. In the presence of symmetrical Rb+ or Cs+, the single channel conductance gsc in WT CNGA1 channels is highly voltage dependent and this voltage dependence is abolished in mutant channels E363A and T364A. CNGA1 channels have structurally-distinct types of voltage sensors, formed by charged and hydrophobic helices. We tested two mutants having a single cysteine in different conditions were different for αB and αC helix mutants. This indicates that ligand binding does not have the same effect on the two connected helices or, in other words, that the conformational change in αB helix is not totally dependent on that in αC helix. Therefore, the primary event in ligand binding is not the αC helix motion. Accordingly, point mutations in a functionally relevant residue in αC helix affected activity of the full-length channel only partially, suggesting that residues outside this helix must be involved in the activation mechanism.

1437-Pos Board B329 Exploring the Mechanism of Cyclic Nucleotide Activation in the MLOTIK1 Potassium Channel
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Cyclic nucleotides are essential elements in the cellular responses to hormones, light and smell. These molecules bind to their receptors through a four-membered cyclic nucleotide binding (CNB) domain, propagating a conformational change to an effector domain. We seek to understand the molecular details of the cyclic nucleotide activation mechanism. Our model protein is the CNB domain of a cyclic nucleotide regulated potassium channel from Mesorhizobium loti, a soil bacterium. This CNB domain is formed by a β-roll and three α-helices, namely αA, αB and αC. A major difference between the bound and unbound states is the relative position of αC helix, which raises the hypothesis that αC helix motion could be the primary event in cyclic nucleotide binding.

To address the hypothesis, we have monitored the conformational change in the isolated CNB domain. We tested two mutants having a single cysteine in αB or αC helix using a cysteine-reacting probe. Reaction kinetics were quantified by determining rate constants, which reflect the relative exposure of the cysteine. The cyclic nucleotides tested were not all equivalent in their rate constants. Moreover, the ratios between rate constants determined in the same conditions were different for αB and αC helix mutants. This indicates that ligand binding does not have the same effect on the two connected helices or, in other words, that the conformational change in αB helix is not totally dependent on that in αC helix. Therefore, the primary event in ligand binding is not the αC helix motion. Accordingly, point mutations in a functionally relevant residue in αC helix affected activity of the full-length channel only partially, suggesting that residues outside this helix must be involved in the activation mechanism.