composed by the called C-linker and the Cyclic Nucleotide Binding Domain (CNBD), both in charge of the gating process after the binding of cyclic nucleotides. In order to understand the gating process, we performed Molecular Dynamic (MD) simulations in presence and absence of cAMP of two structures available in the Protein Data Bank (PDB), which have high sequence homology with CNG channels. In particular, we used one CNBD from M. Loti K^+ channels and, from Hyperpolarization-activated Cyclic Nucleotide-modulated (HCN) channels, we used a monomer and the complete crystallize structure (C-linker/CNBD tetramer). During 10 ns of free MD we observed small differences between CNBD with and without cAMP. On the other hand, C-linker/CNBD suffers a conformational change upon cAMP binding, which could explain how can be exerted force to the pore region. No large differences were observed between the tetramer with or without ligand. Also, we performed several Steered Molecular Dynamics (SMD) of one monomer alone (both, CNBD and C-linker/CNBD) and when it forms part of the tetramer (C-linker/CNBD). We could follow the different unfolding pathways when cAMP is or is not bounded to the CNBD by using differential contact maps.

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Gating of Cyclic Nucleotide Gated Channels is also Voltage Dependent

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Structurally, cyclic nucleotide gated (CNG) channels belong to the family of voltage gated ion channels but their gating is not voltage dependent and it is an open question why CNG channels are voltage-insensitive despite harbouring the usual S4-type voltage sensor. CNG channels are poorly selective and are permeable to several monovalent/divalent cations. Here we show that only in the presence of Li+, Na+ and K+ gating of recombinant WT CNGA1 and native CNG channels is voltage independent, while in the presence of Rb+ and Cs+ and of organic cations their gating is highly voltage dependent. Macroscopic and single channel recordings suggest the existence of two different voltage dependent mechanisms operating together in the presence of Rb+ and Cs+: an inward rectification of the open pore reflecting asymmetries in ion permeation and a voltage and time dependent gating. Mutagenesis experiments show that voltage sensing occurs through a voltage sensor composed of charged/polar residues in the pore and of the S4-type voltage sensor. Neutralization of charged Glu363 in the pore abolishes voltage dependent asymmetries in ionic permeation while neutralization of the second charged Arginine in the S4 transmembrane segment affects voltage dependent gating of the channel. During evolution, CNG channels have lost voltage sensing when Na+ or K+ permeate so that in vertebrate photoreceptors CNG channels are open also at negative voltages, a necessary condition for phototransduction.

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Identification of a Second Binding Site within the Pore of CNGA1 Channels

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Cyclic nucleotide gated (CNG) channels and K+ channels share a significant identity and are thought to share a similar 3D structure. K+ channels can accommodate at the same time several permeating ions inside their pore and are referred as multi-ion channels. Also CNGA1 channels are multi-ion channels, as they exhibit an anomalous mole fraction effect (AMFE) in the presence of mixtures of 110 mM Li+ and Cs+ on the cytoplasmic side of the membrane. Several observations have identified the ring of Glu363 in the outer vestibule of the pore as one of the binding sites within the pore of CNGA1 channels. In the present communication we show also that Cs+ ions at the intracellular side of the membrane, block the entry of Na+ ions. This blockage is almost completely removed at high hyperpolarized voltages as expected if the Cs+ blocking site is located within the transmembrane electric field. Indeed mutagenesis experiments show that the block is mediated by Thr359 and Thr360 at the intracellular entrance of the selectivity filter. In T359A mutant channel AMFE in the presence of intracellular mixtures of Li+ and Cs+ is still present but is reduced or abolished in T360A mutant channel in the presence of 110 mM extracellular Cs+ and Li+ respectively. Therefore the ring of Thr360 at the intracellular entrance of the selectivity filter form another binding site in the CNGA1 channel. These two binding sites are not independent and indeed they mediate a powerful coupling between permeation and gating, specific of CNG channels.

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TRIP8B Allosterically Regulates the Ability of cAMP to Enhance the HCN2 Channel Opening

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The opening of hyperpolarization-activated cyclic nucleotide-gated cation (HCN) channels is enhanced by the binding of cAMP to a cytoplasmic cyclic nucleotide binding domain (CNBD). This action results from an allosteric mechanism in which cAMP binds more tightly to the open versus closed state of the channel, thereby shifting channel opening to more depolarized voltages (quantified by the shift in the midpoint voltage of activation, $\Delta V1/2$). Recently, the brain-specific cytoplasmic protein TRIP8b has been shown to form an auxiliary subunit of HCN channels. In heterologous systems, TRIP8b both alters channel membrane trafficking and inhibits channel opening by shifting the V1/2 to more negative potentials. Previous results indicate that this inhibitory action of TRIP8b results from an antagonism of the ability of cAMP to enhance channel opening. Moreover, the effect of TRIP8b on HCN gating was found to result from the interaction of a central core region of TRIP8b with the CNBD of the channel. Here we ask: Does the effect of TRIP8b involve a direct competition with cAMP for the CNBD, or does it reflect an allosteric action? By creating TRIP8b-HCN2 fusion proteins, we demonstrate that TRIP8b shifts the relation between cAMP concentration and $\Delta V1/2$ to higher cAMP concentrations. Furthermore, we find that direct application of TRIP8bmini, an 80 amino-acid domain in the conserved central core of TRIP8b, to inside-out patches is sufficient to antagonize the action of cAMP similar to that seen with full-length TRIP8b. Moreover, we find that TRIP8bmini reduces the $\Delta V1/2$ observed with saturating levels of cAMP (i.e., decreases ΔV_{max}), suggesting that TRIP8b does not directly compete with cAMP but rather regulates HCN2 channels through an allosteric mechanism, decreasing both the efficacy of cAMP and the sensitivity of the channel to this ligand.

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Rescuing Proper Trafficking of Cysteine Mutant Proteins

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Cysteine substitution has been widely used in structure and function studies, but sometimes mutant proteins get retained during trafficking and the cell is unable to deliver full-length membrane proteins to the cell surface. Similarly, in many inheritable genetic diseases cysteine mutant proteins encounter the same fate. In the visual system, for example, some cysteine mutants of cyclic nucleotide-gated channel (CNG channels) are retained in the ER, leading to achromatopsia (color blindness). We reasoned that it should be possible to modify the chemical structure of the mutation in order to mimic the side chain of the wild type amino acid and recover proper trafficking. As proof of principle, we have studied two naturally occurring cysteine mutants (Y181C and R277C in CNGA3). These mutations are responsible for hereditary cone photoreceptor disorders. We introduced both achromatopsia-related cysteines in a cysteine-less CNG channel, and used them as targets for specific chemical modification with hydroxybenzyl-(MTSHB) and aminoethyl-methanethiosulfonate (MTSEA). These reagents readily attach to the side chain of cysteines and mimic the chemistry of tyrosine and arginine, respectively. Cell surface expression was assayed in *Xenopus* oocytes using fluorescence microscopy and electrophysiology. We successfully restored trafficking and normal function to CNG mutant channels $R \rightarrow C$ and $Y \rightarrow C$, as well as three more cysteine mutants within the S4 transmembrane segment that are known to be retained in the ER. This chemical method provides a unique opportunity to functionally characterize previously inaccessible proteins, as well as it can be readily implemented to assess the chemical nature of misfolding problems, conformational dynamics of folding processes or to study protein conformational changes at the site of the cysteine mutation.

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Functional Role of the Modulatory Subunits in the Olfactory CNG Channels

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Cyclic nucleotide-gated (CNG) channels of olfactory neurons are heterotetramers composed of three homologue subunits, 2xCNGA2, CNGA4 and CNGB1b. CNGA4 and CNGB1b subunits contribute to functional channels only in heterotetramers with CNGA2 subunits but not when expressed alone. It is well established that cAMP and cGMP can activate homotetrameric CNGA2 channels by binding to the cyclic nucleotide-binding domain (CNBD) and that the presence of the CNGA4 and CNGB1b subunit in heterotetrameric channels leads to an increased apparent affinity for the cyclic nucleotides. Our main question is: Do the CNGA4 and CNGB1b contribute to channel opening by binding cyclic nucleotides or only through allosteric modulation? By expressing TFP-labelled CNGA4 and CNGB1b subunits in