3673-Pos
Mechanism of Regulation of Kef Channels by Chemically Diverse Glutathione Molecules
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KTN (RCK) domains are ubiquitous, canonically dimeric, cytoplasmic regulatory domains that control the flux of K^+ transporters and channels in response to cellular cues. We have determined the structures of the C-terminal KTN-bearing domain of KefC, bound alternately to the inhibitory ligand, reduced glutathione, or an activating glutathione adduct. Analysis of these structures reveals that the former stabilizes an inter-domain association between helix α2 from one KTN domain with helices α7/8 of the partnering chain. In contrast, activating glutathione conjugates disrupt this interaction. The resulting conformational change directly impacts the physical exposure of the KTN protein interaction interface that is critical to controlling ion flow through the pore. The elucidated mechanism explains both how subtle chemical differences in glutathione derivatives can have contrary affects on transporter function, as well as how chemically diverse adducts can all elicit activation of this system.

Cyclic Nucleotide-gated Channels

3674-Pos
Ligand Binding and Activation Gating in CNGA2A4B1b Channels
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The olfactory cyclic nucleotide-gated (CNG) channels are heterotetramers composed of three homologue subunits, CNGA2, CNGA4, CNGB1b. The CNGA4 and CNGB1b subunits produce functional channels only in heterotetramers with CNGA2. The channels are activated by cyclic nucleotides as cAMP or cGMP. So far the contribution of the different subunits to channel activation has not been elucidated. It is even not known whether the ligands bind to the CNGA2 subunits only or also to the CNGA4 and CNGB1b subunit, because both have a cyclic nucleotide binding domain in their C-terminus.
CNGA2A4B1b channels were expressed in *Xenopus* oocytes and they were studied in excised patches by monitoring ligand binding and gating under both steady-state and non-steady state conditions. Ligand binding was measured by confocal patch-clamp fluorometry using a fluorescent cGMP analogue (fcGMP). Similar to the homotetrameric CNGA2 channels, we observed also in heterotetrameric channels a crossover of the normalized steady-state binding and steady-state activation. Moreover, the binding curve of the CNGA2A4B1b channels closely approximates the binding curve of the homotetrameric channels.
In order to study the binding to the β subunits we used two approaches: first we expressed either CNGA4 or CNGB1b alone in oocytes and secondly we coexpressed either CNGA4 or CNGB1b with a mutated CNGA2 subunit that has decreased sensitivity to cGMP. Using patch-clamp fluorometry with either fcGMP or fcAMP no significant binding was observed to the CNGA4 and CNGB1b subunits. Furthermore, activation time courses following [cGMP] or [cAMP] jumps were considered. The cGMP-induced activation of CNGA2A4B1b channels was up to 5 times slower than that by cAMP over a wide concentration range whereas in CNGA2 channels they were similar.
Our results seem to suggest that CNGA2A4B1b channels are activated by ligand binding to the two CNGA2 subunits only. Other possibilities are discussed.

3675-Pos
Site-Specific Protein Labeling using Reversible Transition Metal Ion Binding
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Fluorescence spectroscopy is a powerful tool for studying the structure and conformational dynamics of protein molecules both in isolation and in their cellular context. Fluorescence experiments frequently employ small, cysteine-reactive fluorophores. However, it can be difficult to obtain specific labeling of a desired cysteine in proteins with multiple cysteines or in a protein's native environment in which many cysteine-containing proteins are present. To obtain specific labeling, we have developed a method where a desired cysteine can be reversibly protected by binding transition metal ions (e.g. Cd^{2+} and Zn^{2+}) while background cysteines are blocked with non-fluorescent covalent modifiers. Following removal of transition metal ions, the deprotected cysteine is then available to specifically react with a fluorophore. In order to protect specific cysteines, the affinity for metal binding was increased by placing metal binding residues nearby in regions of known secondary structure (e.g. an alpha-helix) to act as coordination partners in a metal binding site. The placement of histidine residues next to or one turn away from a cysteine in an alpha-helix increased the metal binding affinity of that cysteine. This allows for protection of a particular cysteine at much lower metal concentrations. Using pairs of cysteines, rather than a combination of cysteines and histidines, further stabilized metal binding. Finally, we demonstrate the ability of this technique to selectively label a particular cysteine in mixtures of proteins containing cysteines. These motifs are simple, can be introduced into proteins with minimal perturbation, and allow for selective labeling of sites within a protein, specific proteins in a mixture, or proteins in a native environment. This technique has great potential for use in patch-clamp fluorometry studies of ion channels in which non-specific labeling of patch-associated proteins makes it difficult to resolve fluorescence from channel proteins.

3676-Pos
Cyclic Nucleotide Gated Channels have a Functional Voltage Sensor Disabled by Hydrophobic Interactions
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Voltage-gated Na+, K+, Ca2+ and cyclic nucleotide-gated (CNG) channels belong to the same super-family of ion channels. All these channels harbour a voltage sensor but their gating is different: the opening of CNG channels is poorly dependent on membrane voltage, whereas the gating of Na+, K+, Ca2+ channels is highly voltage-dependent. By using electrical recordings in voltage clamp mode, we show that in wild type CNGA1 channels the voltage sensor does not move as in K+ channels. However, when the hydrophobic interaction coupling Phe380 in S6 to Leu356 in the P-helix is impaired, the voltage sensor moves as in K+ channels and the gating of mutant channels F380A and L356A becomes voltage dependent. Voltage gating in mutant channels F380A is accompanied by nonlinear capacitative transients at the beginning and termination of voltage stimuli, reminiscent of gating currents measured in Kv channels, suggesting dipole rearrangement or charge translocation caused by the applied electrical field. Moreover, when Arg272 and Arg275 in the S4 segment are neutralized one at time in a F380A background, significant changes of the characteristic activation parameters can be observed, in agreement with what described for Kv and Nav channels. Therefore, we can conclude that the voltage sensor in CNG channels is blocked by the tight coupling between the P-helix and S6, necessary condition that makes the channel gated by binding of cyclic nucleotides and not by changes of membrane voltage.

3677-Pos
Interaction Energies between Intracellular Regions in CNG Channel Activation
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Cyclic nucleotide-gated (CNG) channels mediate sensory signal transduction in retinal and olfactory cells. The channels are activated by the binding of cyclic nucleotides to an intracellular cyclic nucleotide-binding domain (CNBD). The molecular events translating the binding to the pore opening are still unknown. We investigated the role of intracellular channel regions on the activation process by constructing chimeric channels in which the N-terminus, the S4-S5 linker, the C-terminus, and the CNBD of the retinal CNGA1 subunit were systematically replaced by respective regions of the olfactory CNGA2 subunit. Macroscopic concentration-response relations were analyzed, yielding the apparent affinity to cGMP and the Hill coefficient. The degree of functional coupling of intracellular regions in the activation gating was determined by an interaction energy according to the principles of thermodynamic double-mutant cycle analysis. We show that all four intracellular regions, including the short S4-S5 linker, are involved in controlling the apparent affinity of the channel to cGMP and, moreover, in determining the degree of cooperativity between the subunits as determined from the Hill coefficients. The interaction energies are specific for pairs of regions. The interaction energy of the S4-S5 region with

both the N-terminus and the C-linker was significantly different from zero at all possible combinations of intracellular regions. In contrast, at all possible combinations of intracellular regions no significant interaction energy was observed for the S4-S5 linker and the CNBD. It is concluded that in CNG channels the S4-S5 linker cooperates with both N-terminus and C-linker in the process of translating ligand binding to the pore opening.

3678-Pos

Structural Insight into the Ion Selectivity and Ca^{2+} Blockage in Cyclic Nucleotide Gated Channels

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Cyclic nucleotide-gated (CNG) channels are non-selective cation channels that play crucial roles in visual and olfactory signal transduction. They conduct all alkali metal and some alkaline earth metal ions, most notably Ca^{2+}. In this study the non-selective bacterial channel NaK is used as a model to study the structural basis of ion selectivity in CNG channels. Though NaK is non-selective and bears some similarities with CNG channels, there is divergence in some of the most critical properties of CNG channels. Moreover, the protein sequence at the C-terminal end of the selectivity filter in NaK differs markedly from CNG channels both in amino acid composition and sequence length. As a result, wild-type NaK may not be a viable structural model for CNG channel selectivity. So, within the NaK selectivity filter (^{63}TVGDGNFS^{70}) the DGNFS sequence was replaced with ETPP to represent most CNG channels, DTPP to mimic an E◇D mutation and NTPP to represent a neutral residue mutant. We present high resolution crystal structures and single channel recordings of of these NaK channel mutants. These mutants share several striking functional similarities in ion selectivity with eukaryotic CNG channels: they are non-selective and permeate Na^+ and K^+ equally well; externally added Ca^{2+} serves as a permeating blocker, with the conserved acidic residue in the filter mediating Ca^{2+} binding. The structures of these CNG-mimicking mutant channels in complex with various cations reveal a unique selectivity filter architecture containing three contiguous ion binding sites different from both wild type NaK and K^+ channels. Taking into account identical selectivity filter sequences, these structures are believed to serve as accurate working models for CNG channel pores and yield novel insights into the structural basis of their ion selectivity and Ca^{2+} blockage properties.

3679-Pos

Subunit-Specific Regulation of Photoreceptor CNG Channels by Phosphoinositides

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Cyclic nucleotide gated (CNG) channels in retinal photoreceptor cells play a key role in vertebrate phototransduction. The ligand sensitivity of photoreceptor CNG channels is adjusted during adaptation and in response to paracrine signals, but the mechanisms involved in channel regulation are only partly understood. Heteromeric A3+B3 (cone) and A1+B1 (rod) channels are sensitive to regulation by PIP_3 or PIP_2, demonstrating a decrease in apparent affinity for cGMP. To determine what subunit types are necessary for PIP_3 sensitivity, we generated heteromeric channels by co-expression of PIP_3-insensitive A2ΔN (Brady et al., 2006) with B3 or B1 subunits. Using patch-clamp recording in the inside-out configuration, we found that both channel types were insensitive to PIP_3 regulation, suggesting that A3 or A1, but not B3 or B1 subunits, confer phosphoinositide sensitivity to heteromeric channels. Consistent with this idea, co-expression of A3 with B1 formed channels that were sensitive to PIP_3 regulation. Unlike homomeric A1 or A2 channels, A3-only channels paradoxically did not show a decrease in apparent affinity for cGMP after PIP_3. However, PIP_3 induced a nearly three-fold increase in cAMP efficacy for A3 channels, an effect that was reversed by poly-lysine application. The PIP_3-dependent increase in cAMP efficacy for A3 channels was abolished by mutation of a critical ligand-discrimination residue (D609K) or by truncation of the channel distal to the cyclic nucleotide-binding domain (613stop). Furthermore, the apparent cGMP affinity of A3-613stop channels was reduced three-fold by PIP_3; this change in cGMP sensitivity also was reversed by poly-lysine. Together, these results suggest that regulation of A3 subunits by PIP_3 exhibits two components, one of which is unmasked either by assembly with B3 subunits or by deletion of the C-terminal region of A3. (Supported by NIH EY012836)

3680-Pos

Transferring S3-S4 Motif from Kv Channels to the CNG Channel

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Cyclic nucleotide-gate (CNG) channels belong, functionally, to the family of ligand-gated ion channels. They are activated by the binding of cyclic nucleotides, such as cGMP, cIMP or cAMP to an intracellular binding domain within the carboxyl terminal. Structurally, however, CNG channels are grouped with ion channels containing six transmembrane segments, such as voltage-dependent potassium channels. These proteins probably derive from a common evolutionary ancestor, but the properties of the channels are quite different. Whereas the membrane potential controls the activity of voltage-gated K^+ (Kv) channels, CNG channels have little or no inherent voltage dependence, particularly at saturating concentrations of agonist. Even though CNG channels contain an S4 positively-charged segment similar to the S4 voltage sensor in K_V channels, little is known about its relevance in CNG channels. In this work, we transferred the voltage-sensor S3-S4 motif from Kv2.1 and KvAP to bCNGA1 and analyzed the function of these chimeras expressed in *Xenopus* oocytes using excised patch clamp. Our results suggest that the S3-S4 motif of CNG channel can be swapped by the homologous region of Kv channels and, although these chimeras are modulated by voltage, activation remains cGMP-dependent.

3681-Pos

Caesium Permeation Reveals an Unusual Voltage Dependent Gating at the Selectivity Filter of CNGA1 Channels

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CNG channels are permeable to alkali monovalent and divalent cations and to small organic cations. Therefore, CNG channels have a low ionic selectivity, attributed to an intrinsic flexibility of the filter region (Laio & Torre, 1999) mediating the coupling between permeation and gating (Gamel & Torre 2000; Holmgren, 2003; Kush, 2004). We have analysed in more detail the permeation of Cs+ in WT CNGA1 channels. In symmetrical Na+ or K+ conditions and in the presence of saturating cGMP concentrations the ratio between the current at +200 and −200 mV I200/I-200 is 1.3. In contrast, in the presence of symmetrical Cs+ I200/I-200 it is about 0.75. Under these conditions, single channel recordings reveal a surprising behaviour: the single channel conductance for Cs+ ions is about 18 pS at −180 mV, but becomes less than 5 pS at voltages larger than 100 mV. The open probability at −180 mV is about 0.2 and becomes close to 1 at +100 mV. When Thr360 is mutated to alanine, the single channel conductance for Cs+ ions is around 15 pS both at +100 and −100 mV. These results confirm the notion that the pore region of CNGA1 channels is highly flexible and that permeating ions modify and control channel gating. These results show also that the pore region of CNGA1 channels acts as a voltage sensor and modifies their conformation in response to changes of the applied field. The molecular mechanisms involved in these rearrangements are likely to consist in reorientation of electric dipoles, such as those of Thr360.

3682-Pos

Functional Expression and Subcellular Localization of F-Channels in Human Ventricular and hESC-Derived Cardiomyocytes

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Caveolae are specialized lipid rafts in the plasma membrane responsible for the interaction, sublocalization and function of proteins and ion channels. The Hyperpolarization-activated Cyclic Nucleotide-gated (HCN) genes encode for the alpha subunit of f-channel present in cardiac pacemaker cells, in the adult and developing cardiac myocytes (CM). HCN4 is the predominant isoform in the sinoatrial node and, in the rabbit, experimental evidence indicate that it localizes into membrane caveolae, where caveolin-3 (Cav3)-channel interaction regulates current properties and autonomic modulation. HCN4 is abundant in undifferentiated human embryonic stem cells (hESC) and immature hESC-derived CM (hESC-CM). To date, no information is available on i) developmental changes of HCN4 channel localization and function in human CM ii) the relationship with HCN channel expression/function in adult CM.
Confocal analysis showed that HCN4 and Cav3 colocalize in adult human ventricular CM. In the same cells, f-current was consistently recorded upon hyperpolarization (70% cells), with a voltage of half maximal activation (Vh) of −94 mV. Protein and mRNA for Cav3 were not detected in undifferentiated hESC, but expression increased during maturation of hESC-CM. Oppositely, HCN4 was highly expressed in hESC and early hESC-CM, but a 5-fold decrease in mRNA levels occurred in late hESC-CM. In these cells, HCN4 appeared to be associated with Cav3. Activation properties of f-current recorded in late hESC-CM resembled those measured in adult ventricular CM (Vh=-93 mV). Current activation was faster and occurred at more positive potentials in hESC and early CM. In conclusion, cardiac maturation is associated with the recruitment of HCN4 channel and CAV3 into membrane lipid rafts, suggesting that sub-cellular localization of f-channel in lipid rafts is a fundamental step during cardiac maturation.