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Symposium 1: Ion Channel Gating

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The domains in the Na channel have specific functions

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The classical voltage-dependent sodium channel is responsible for the upstroke of the action potential. Its pore forming subunit is a single polypeptide that has four homologous domains. Although each one of these domains is similar to a single subunit of the voltage gated potassium channel, the structures differ among each other. For example, the number of basic residues in S4 segments, responsible for voltage-sensing, is different in each domain. We have probed the function of each domain in the overall operation of the channel using fluorescent tags because they can detect local conformational changes. The results indicate that the S4 segments of domains I, II and III have faster kinetics than the S4 segment of domain IV. The kinetics and voltage dependence as reported by the fluorescent probe in the first three domains agree well with that of the fast component of the gating currents. On the other hand, the kinetics of the fluorescence probe attached in S4 of domain IV matches the kinetics of the slow component of the gating current. In addition, the turn-on of the fluorescence of domain IV exhibits a lag that is not observed in the fluorescence of the first three domains, indicating that the movement of S4-DIV does not start until one of the other three S4's has moved. The kinetics of S4-DIV (or the slow component of the gating current) is too slow to account for the activation of the conductance but is faster that the time course of inactivation. In fact, the ionic current develops before the fluorescence change in S4-DIV. Taken together, these results indicate that the first three domains are responsible for the activation of the conductance and suggest that S4-DIV does not participate in channel opening. Other correlations of the fluorescence of S4-DIV with the kinetics and steady-state properties of inactivation indicate that the function of S4-DIV is related to the voltage dependence of inactivation that is ultimately produced by the IFM motif that blocks ion conduction.

The local detection of conformation by the fluorescent probe can also be used to test possible interactions between domains during the operation of the channel. Thus, by introducing a mutation in one domain that affects the voltage dependence of the movement of that domain, one can ask whether that mutation has an effect in the movement of another domain. We have found that in fact all domains interact with each other with positive cooperativity. It can be demonstrated that positive cooperativity among voltage sensors increases the overall kinetics of channel opening. These results provide at least a partial explanation as to why sodium channels are so much faster than potassium channels, a requirement to generate the action potential.

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Modulation of potassium channel conformation and function by permeating ions

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The Kv2.1 potassium channel is a very slowly inactivating delayed rectifier, with widespread distribution in brain, peripheral neurons and excitable cells such as heart and pancreas. In hippocampal neurons, where its function has been best studied, it appears to be profoundly important under conditions of high frequency firing or elevated extracellular [K^+]. For example, with physiologically relevant elevation of extracellular [K^+], action potential duration is increased ~10 fold in the absence of Kv2.1 function, yet is unaffected in the presence of normal Kv2.1 function (Du *et al.*, 2000). In contrast, Kv2.1 appears to have little importance to the integrity of single action potentials under physiological conditions. We have studied the molecular mechanism by which this seemingly standard delayed rectifier performs this very specific function. Kv2.1 channels open into one of two conformations. This conformational difference influences activation rate, inactivation rate, current magnitude and channel pharmacology. All of the functional effects of this difference in conformation are related to the orientation of a single outer vestibule lysine. In one conformation, currents are bigger and activate faster, whereas in the other, currents are smaller and activate more slowly. Which conformation the channel opens into appears to be determined by the occupancy of a particular K^+ binding site in the channel's selectivity filter. Thus, with elevation of [K^+], occupancy of this site is greater, and channels open into a conformation that produces a larger current. In addition, two outer vestibule lysines dramatically reduce current magnitude variation associated with changes in K^+ driving force that accompany changes in extracellular [K^+]. Together, these two mechanisms produce the unique phenotype of the Kv2.1 channel. Upon elevation of external [K^+], current density through Kv2.1 is increased at all membrane potentials, whereas in all other K^+ channels, it is reduced. We propose that this increase in outward current density acts to maintain action potential integrity in the face of elevated extracellular [K^+], which occurs during high frequency firing. From a biophysical perspective, the Kv2.1 channel displays another apparently unique mechanism. One of the fundamental mysteries in ion channel biophysics, which has potentially significant clinical importance, is how single channel conductance is controlled. Recent experimental and theoretical studies suggest that, in many K^+ channels, single channel conductance is determined by inner vestibule characteristics. In contrast, our data demonstrate that single channel conductance in Kv2.1 can be modulated by reorientation of the outer vestibule. Thus, it may be that targets of conductance modulation will be different in different K^+ channels.

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The gating of mechanosensitive ion channels

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Mechanosensitive (MS) ion channels are a special type of integral membrane proteins activated by membrane deformation caused by mechanical stimuli experienced by living cells. They convert mechanical stimuli into electrical and/or chemical intracellular signals. There is a great diversity of these channels in terms of ionic conductance, selectivity or voltage dependence. These channels have been found in all types of prokaryotic and eukaryotic cells. In animals and humans they play a role in hearing, touch, proprioception or regulation of blood pressure. In plants they may function as gravity sensors in gravitropism, whereas in bacteria they constitute a mechanism that prevents excessive water inflow and build-up of excessive turgor pressure by acting as mechano-electrical switches, which open in response to cell membrane deformations caused by osmotic forces under hypotonic conditions. Among the MS channels studied to date the best characterised are bacterial MscL and MscS channels, the MS channels of large (L) and small (S) conductance (Martinac, 2004). Their 3D structure was determined by X-ray crystallography allowing for in-depth studies of the gating mechanism in these channels. In particular, the structure, function and structural dynamics of MscL channel has been well characterized by a number of techniques including the patch-clamp technique, electronparamagnetic resonance (EPR) spectroscopy, molecular dynamics simulations and most recently FRET spectroscopy. MscL and other prokaryotic MS channels are gated by bilayer deformation forces indicating that mechanism of mechanotransduction in these channels is defined by both local and global asymmetries in the transbilayer pressure profile and/or bilayer curvature at the lipid protein interface (Perozo *et al.*, 2002a, 2002b). Moreover, eukaryotic MS ion channels found in non-specialized mechanotransducer cells, such as TREK-1 (Patel *et al.*, 2001) and TRPC1 (Maroto *et al.*, 2005), have also been shown to be gated by membrane tension purely developed in the lipid bilayer. The implication of these findings is that the lipid bilayer is much more than a neutral solvent by actively modulating the specificity and fidelity of signalling by membrane proteins.

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The mechanism of fast gating in ClC chloride channels

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ClC proteins are a ubiquitous family of chloride channels and transporters that perform diverse functions such as the stabilisation of membrane potentials and the regulation of cell volumes. The most widely studied member of this family is ClC-0 from the *Torpedo* electric ray that has been shown to contain two independent ion conductive pores and have two distinct voltage gating mechanisms. The 'slow' or inactivation gate operates on both pores in the dimer simultaneously, whereas the 'fast' gate acts on each pore individually and opens and closes at a much faster rate. We have investigated the hypothesis that the side chain of a single glutamate residue acts as the fast gate in these channels using molecular dynamics simulations. We find that the motion of this side chain can indeed gate the channel, and furthermore demonstrate that this mechanism explains the dependence of channel gating on extracellular Cl^- concentration, membrane potential and pH.

Using the crystal structure of a bacterial ClC protein as a template we first create a putative open state configuration of the ClC-0 channel. Using this open state model we then conduct molecular dynamics simulations to study the motion of the central glutamate side chain. We find that when the side chain extends towards the extracellular end of the channel it presents an electrostatic barrier to Cl^- conduction. However, external Cl^- can push the side chain into a more central position where, pressed against the channel wall, it does not impede the motion of Cl^- ions. Alternatively, the barrier to ion conduction can be removed by a proton from a low pH external solution binding to the side chain and neutralising its charge. Finally we use Brownian dynamics simulations to demonstrate the influence of membrane potential and external Cl^- concentration on the open probability of the channel.

Conformational changes associated with glycine receptor activation

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The glycine receptor Cl^- channel (GlyR) mediates inhibitory neurotransmission in the central nervous system. Like other members of the ligand-gated ion channel family, functional GlyRs comprise 5 subunits arranged symmetrically around an ion-conducting pore. Each subunit consists of a large external ligand-binding domain and 4 α-helical transmembrane domains (M1-M4). The external M2-M3 linker domain is crucial for transmitting the agonist-induced conformational change to the channel gate. Consistent with this role, a substituted cystine accessibility study on the M2-M3 linker of the α1 GlyR showed that the surface accessibility of 6 contiguous cysteine-substituted residues (R271C to K276C) was increased in the open state (Lynch *et al.*, 2001). Thus, the conformation of the M2-M3 domain depends on the degree to which the GlyR is activated by agonist. This study investigated whether the closure of the channels by picrotoxin preserves the relationship between domain conformation and fractional peak current magnitude that is seen in its absence. If this relationship is not preserved, it may be concluded that picrotoxin closes the channel by inducing a novel conformational change in this domain.

HEK293 cells were transfected with WT and mutant GlyR cDNA using the calcium phosphate precipitation method. Transfection solution was removed after 24 h and glycine-gated currents were recorded using whole-cell patch clamp techniques over the following 24−72 h. The surface accessibility of the introduced cysteines was assessed via their reaction rate with the sulfhydryl modifying agent, methanethiosulfonate ethyltrimethylammonium (MTSET) as previously described (Lynch *et al.*, 2001).

Picrotoxin significantly slowed the reaction rate of MTSET with A272C, S273C and L274C, although it had no measurable effect on R271C, P275C or K276C. Before interpreting this result as a picrotoxin-specific conformational change, it was necessary to eliminate the possibility of steric competition between picrotoxin and MTSET. One way of achieving this is to identify the location of the picrotoxin binding site. Accordingly, we showed that picrotoxin and the structurally-unrelated pore blocker, bilobalide, were both trapped in the R271C GlyR in the closed state and that a point mutation to the pore-lining T6' residue abolished inhibition by both compounds. We also demonstrated that the picrotoxin dissociation rate was linearly related to the channel open probability. These observations constitute a strong case for picrotoxin binding in the pore.

By binding in the pore, picrotoxin cannot sterically hinder MTSET from reacting with M2-M3 domain cysteines. We therefore conclude that picrotoxin changes the MTSET reaction rate by changing the intrinsic reactivity rates of the introduced cysteines. Because PTX changes the relationship between equivalent concentration and cysteine reactivity, we conclude that it alters the conformation of the GlyR M2-M3 domain in a way that cannot be achieved by simply varying the glycine concentration alone. This result implies that the M2-M3 domain integrates information from multiple categories of binding sites and sends a net signal to the activation gate. This reveals a hitherto unexpected complexity in the role of the M2-M3 domain.

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