internal Ca^{2+} by patch clamp on inside-out configuration. Gating currents were elicited by steps of 1 ms to increasing voltages ranging from −90 to 350 mV in increments of 10 mV, and integrated during the onset of depolarizing pulses to generate charge versus voltage (Q/V) curves. Our results show that the effects of the β1 subunit on the resting-active equilibrium of the voltage sensor is determined by the N- and C-terminal with a major contribution of the N-terminus.

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Whole-Cell Gating-Charge Measurements for Analysis of Allosteric Domain Coupling in Hslo1 BK Channels

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Large-conductance calcium- and voltage-activated potassium (Slo1 BK) channels are important for smooth muscle contraction and neurotransmitter release. Since BK channel gating is allosteric in nature, the conformations of the domains sensing transmembrane voltage (VSD) and intracellular calcium (CSD) reciprocally influence each other, either directly or via the central pore/gate domain (PGD). Thus, to fully understand the gating of BK channels involving multiple domains, determination of voltage dependence of both conductance (GV) and gating-charge movement (QV) is required. Here we evaluated how VSD-linker segments facing the CSD influence channel gating. Whole-cell gating currents were measured upon BK expression in tsa201 cells. In the absence of intracellular Ca^{2+}, the voltage for half-maximal off-gating charge movement ($V_{0.5,Q}$) was 159 ± 2 mV (n=6) with a corresponding apparent gating charge (Q_{app}) of 0.80 ± 0.02 e_0. Coexpression of the human β1 subunit or increasing intracellular [Ca^{2+}] to 10 μM left-shifted $V_{0.5,Q}$ by 42 mV and 70 mV, respectively, without significant changes in Q_{app}. To investigate allosteric domain interactions in BK, we analyzed the effect of combining mutations within S4/S5 and S6/C linkers on GV and QV in the absence of Ca^{2+}. S6/C-linker mutations G327F and K330F shifted the GV by 109 mV and −133 mV and the QV by 7 mV and −10 mV, respectively. S4/S5-linker mutations F223A+L224A and E219R shifted the GV by −22 mV and 120 mV, and the QV by −97 mV and −109 mV, respectively. In the background of G327F or K330F, GVs were further shifted by up to −200 mV only by F223A+L224A. The data suggest that residues F223 and L224 (S4/S5 linker) and G327 and K330 (S6/C linker) are involved in allosteric coupling in BK channels. Supported in part by DFG HE 2993/8 and NIH.

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Probing the Voltage Gated Proton Channel Hv1 with FRET

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The Hv1 proton channel has been shown to contribute to acid extrusion after acid loading in cells, it is involved in pH regulation in the airway epithelium and in the innate immune system during the respiratory burst and it has been proposed to play a role in the capacitation process of the sperm, aditionally the proton channel can also help set the membrane potential. The Hv1 channel is similar to the voltage sensing domain of voltage gated K^+ and Na^+ channels, it assembles into a dimer were each of the subunits forms a permeation pathway which is highly cooperative. Here, by measuring Foster Resonance Energy Transfer (FRET) between fluorescent proteins and with the non-fluorescent molecule dipicrylamine (DPA), we attempt to get some insigths about the organization and function of the Hv1 proton channel.

mCitrine or mCerulean fluorescent proteins were attached to the C-termini of the channel. HEK 293 cells were cotransfected with the constructs and FRET was measured by the spectral FRET method. DPA was used as an energy acceptor for the mCitrine fluorescence in injected Xenopus oocytes membrane sheets. DPA intercalates in the membrane, quenching the fluorescence by a FRET mechanisms, allowing distance measurements between the membrane and the C-termini.

We have been able to determine the relative separation between the C-termini of the dimer and its relation to the membrane plane. Results are consistent with the X-ray structure of the coiled-coiled C-terminal domain.

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The Activation Kinetics of the Voltage-Gated Proton Channel is Drastically Accelerated by Unsaturated Fatty Acids

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The unsaturated fatty acids are important component of the biological membranes and the precursors of mediators of inter- and intra-cellular signaling. It is well-known that the unsaturated fatty acids, including arachidonic acid (AA, C20:4), modulate functions of various ion channels in a distinct manner; some ion channels are activated whereas others are inhibited. The voltage-gated proton channel (VSOP/Hv1) can control the proton conductance by membrane voltage and pH. It helps the production of reactive oxygen species by NADPH oxidase in immunocytes. The enhancement of the production of reactive oxygen species by AA has been reported to be accompanied by the increase of Hv currents in neutrophils, macrophages, and eosinophils. However, the detailed molecular mechanisms of actions of AA on the voltage-gated proton channel have remained elusive. Here we report the effects of AA on mouse Hv1 heterologously expressed in HEK293T cells by electrophysiological analysis. We measured Hv currents in the inside-out patch clamp configuration under pH 7.0 in both solutions of pipette and bath. The addition of 50 uM AA with rapid-perfusion system immediately increased the magnitude of Hv currents through mHv1 that are evoked during one second depolarizing step to 100 mV by 20 times. After washout of AA, the currents rapidly returned to the original current level. The analysis with sixty second depolarizing pulse showed that Hv currents exhibited more than 15 times faster activation kinetics and 1.5 times larger steady-state conductance upon the addition of AA. We also examined the effects of other fatty acids and constructs of mHv1. Based on these results, we would discuss the molecular mechanisms and the essential sites for the actions of unsaturated fatty acids on Hv1.

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Electrostatic Interactions in the Closed and Open States of Voltage-Gated Proton Channels

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Voltage-gated proton channels (Hv1) play important roles in the respiratory burst, in pH regulation, in spermatozoa, in apoptosis, and in cancer metastasis. Unlike other voltage-gated cation channels, the Hv1 channel lacks a centrally located pore formed by the assembly of subunits. Instead, the proton permeation pathway in the Hv1 channel is within the voltage-sensing domain of each subunit. The gating mechanism of this pathway is still unclear. Mutagenic and fluorescence studies suggest that the fourth transmembrane segment, S4, functions as the voltage sensor and that there is an outward movement of S4 during channel activation. Using thermodynamic mutant cycle analysis, we find that the conserved positively charged residues in S4 are stabilized by counter charges in the other transmembrane segments both in the closed and open states. We construct models of both the closed and open states of Hv1 that are consistent with the mutant cycle analysis. These structural models suggest that electrostatic interactions between transmembrane segments in the closed state pull hydrophobic residues together to form a hydrophobic plug in the center of the voltage-sensing domain. Outward movement of S4 removes the hydrophobic plug, as if this hydrophobic plug works as the gate that prevents protons to permeate the Hv1 channel in the closed state.

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On the Location of Binding Sites of 2-Guanidinobenzimidazole in the Voltage-Gated Proton Channel

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Voltage-gated proton channels (Hv1) play important roles in the respiratory burst, in pH regulation, in spermatozoa, in apoptosis, and in cancer metastasis. The ability to block them selectively is an important target for drug development and for biophysical studies of channel function. Recently, 2-guanidobenzimidazole (2-GBI), a selective and state-dependent blocker of Hv1 channels was identified. However, the exact location of the binding site for a blocker as well as its apparent state-dependence has yet to be established. Recently, we have investigated the structure of the closed and open states of the voltage-gated proton channel through a combination of modeling and experimental analysis. To understand the mode binding and blocking and to hopefully identify the binding pocket, an initial docking study was performed for all of the ligands reported by Hong *et al* (25) as having significant binding affinity with the open channel monomer.

We tested whether our open- and closed-state models could explain the state-dependence of the binding of the guanidine analogue 2-guanidobenzimidazole (2-GBI) from the cytosolic side of the channel. A combination of free-energy simulations and molecular docking established the architecture of the cytosolic binding site for 2-GBI. The site is centered near F198, consistent with the experimental data allowing for interactions between the positively-charged guanidine moiety of 2-GBI and the negatively-charged residues E201, D222, and E219. The aromatic ring of 2-GBI is stabilized by residues in the proximity of F198. In the closed state model, E201, D222, and E219, are occupied by the