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451-Pos Board B251
Orai N-Terminus Plays a Multi-Facetted Role in Orai Gating
Isabella Derler, Judith Bergsmann, Marc Fahrner, Martin Muik, Klaus Groschner, Christoph Romanin.
In immune cells, STIM1 and Orai1 represent the key components of Ca2+ release-activated Ca2+ channels. While the functional role of the Orai C-terminus has been extensively studied, the one of the N-terminus remains elusive. An among all Orai proteins conserved N-terminal region has been elucidated only for Orai1 to couple to STIM1 C-terminus. With respect to the role of this domain we investigated N-terminal deletion mutants of Orai3 which allows two modes of activation, either by store-depletion or by 2-APB. Increasing N-terminal truncations revealed that retention of Orai3 specific fast inactivation requires the presence of the whole conserved region. Store-dependent activation of Orai3 was obtained as long as the membrane adjacent half of this conserved N-terminal domain was present. Stimulation by 2-APB required even less of these conserved residues. While store-operated functional deletion mutants displayed comparable 2-APB stimulated currents as wild-type Orai3, the store-non-responsive ones exhibited smaller 2-APB currents and more positive reversal potentials with increasing truncations. Contrary to Orai3, Orai1 requires a longer portion of the N-terminal conserved region for preserved store-operated activation. In aggregate this conserved domain plays a multi-facetted role with distinct iso-form specific requirements in the gating of Orai channels. (supported by FWF T 466).

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Resting-State Orai1 Diffuses as Homotetramer in the Plasma Membrane of Live Mammalian Cells
Josef Madl, Julian Weghuber, Reinhard Fritsch, Isabella Derler, Marc Fahrner, Irene Frischauf, Barbara Lackner, Christoph Romanin, Gerhard J. Schütz.
The Calcium Release Activated Calcium (CRAC) current is an important calcium entry mechanism in T-cells and mast cells. The essential pore forming subunit of CRAC channels is the plasma membrane protein Orai1. Functional CRAC channels in store-depleted cells are formed by four Orai1 subunits. However, the subunit composition in quiescent cells is still under discussion. Both a tetrameric as well as a dimeric stoichiometry of resting-state Orai1 has been reported. Here we present single molecule fluorescence microscopy of Orai1 which was performed in order to directly visualize the resting-state pore stoichiometry. The protein was fluorescently labeled as fusion construct with monomeric GFP (mGFP) and expressed in a human cell line (T24). In contrast to previously published single molecule studies we exclusively analyzed mobile Orai1 aggregates, which represent the largest fraction of Orai1 in resting-state cells. The subunit composition was studied using a combined approach of photobleaching and single molecule fluorescence brightness analysis. The method allows reducing the density of fluorescently labeled molecules without affecting the labeling stoichiometry, which is used as the direct measure of the Orai1 pore stoichiometry. Density reduction is necessary for the observation of single channels and is achieved by completely photobleaching a defined area within the plasma membrane. Non-bleached Orai1-mGFP aggregates enter the bleached region subsequently by diffusion. The recorded single molecule trajectories were analyzed using brightness distribution fitting and correlation plotting. The obtained results indicate a tetrameric resting-state subunit stoichiometry.

453-Pos Board B253
Subunit Stoichiometry of Human Orai1 and Orai3 in Closed and Open States
Angelo Demuro, Aubin Penna, Olga Safrina, Anna Amcheslavsky, **Michael D. Cahalan**, Ian Parker.
We applied single-molecule photobleaching to investigate the stoichiometry of human Orai1 and Orai3 channels tagged with eGFP and expressed in HEK cells. At low expression, GFP-tagged subunits were detected in TIRF microscopy as single fluorescent spots that decayed in a step-wise manner as individual GFP molecules bleached. By counting the number of photobleaching steps, the number of subunits per channel complex could be deduced. In fixed cells, Orai1 was detected primarily as dimers when expressed alone and as tetramers when co-expressed with the cytosolic STIM1 fragment, C-STIM1, to activate Ca^{2+} influx, as previously found for *Drosophila* Orai with and without activation by C-Stim (Penna et al., 2008, *Nature* 456:116-120). When co-expressed with full-length STIM1, Orai1 was also found to be predominantly dimeric in living cells under resting conditions with Ca^{2+} stores filled. We also investigated Orai3 alone and when activated by either C-STIM1 to form a Ca^{2+}-selective channel in its store-operated mode, or by addition of 2-APB to form a Ca^{2+}-permeable but relatively nonselective cation channel in its STIM1-independent mode. Similar to our observations with Orai1, eGFP-Orai3 alone was detected mostly as dimers under basal conditions, but predominantly as tetramers when co-expressed with C-STIM1. On the other hand, cells expressing only eGFP-Orai3 that were exposed to 2APB before fixation showed a distribution of bleaching steps closely similar to that observed without 2APB, with a predominance of dimers. These results indicate a predominantly dimeric state for Orai3 at rest or when activated by 2-APB, and a tetrameric channel when activated by C-STIM1. We conclude that the human Orai1 and Orai3 channels undergo a dimer-to-tetramer transition to form a Ca^{2+}-selective pore during store-operated activation, and that Orai3 forms a dimeric non-selective cation pore upon activation by 2-APB.

454-Pos Board B254
Reciprocal Regulation of Store-Dependent and Independent CRAC Channel Gating by STIM1 and 2-Apb
Megumi Yamashita, Agila Somasundaram, Murali Prakriya.
Ca2+ release-activated Ca2+ (CRAC) channels generate Ca2+ signals important for gene expression, the release of inflammatory mediators, and cell motility. Under physiological conditions, CRAC channels are activated by depletion of ER Ca2+ stores via physical interactions with the ER Ca2+ sensor, STIM1. However, recent studies indicate that exogenously over-expressed Orai3 CRAC channels can also be directly activated in a store- and STIM1-independent fashion by the compound, 2-aminoethyldiphenyl borate (2-APB). Whether 2-APB also regulates store-operated gating of Orai3 channels and if STIM1 can modulate direct activation by 2-APB is unknown. Here, we report that 2-APB regulates STIM1 gating of Orai3 channels in several respects. Low concentrations potentiate Orai3 currents in a store- and STIM1-dependent manner, whereas high doses strongly inhibit store-operated Orai3 currents. Inhibition by 2-APB occurs concurrently with changes in several STIM1-dependent Orai3 channel properties, including fast inactivation and potentiation, suggesting that high doses of 2-APB affect STIM1-Orai3 coupling. Conversely, STIM1-bound Orai3 (and Orai1) channels resist direct activation by 2-APB. The resistance to 2-APB activation varies in direct proportion to 2-APB-induced inhibition of the store-operated Orai3 conductance, suggesting that 2-APB has to first uncouple STIM1 from Orai3 channels before causing direct activation. Collectively, our results indicate that the store-dependent and independent modes of CRAC channel activation are reciprocally regulated, such that channels bound to STIM1 resist 2-APB gating, while 2-APB suppresses STIM1-gating.

455-Pos Board B255
STIM1 Regulates CRAC Channel Properties
Beth McNally, Agila Somasundaram, Murali Prakriya.
Store-operated calcium release-activated Ca2+ (CRAC) channels mediate important functions in immune cells including gene expression, the release of inflammatory mediators, and motility of thymocytes. Orai1, a prototypic CRAC channel, exhibits a biophysical fingerprint consisting of an extremely high selectivity for Ca2+, a narrow pore along the permeation pathway, and a very small unitary conductance. It is known that activation of CRAC channels following ER Ca2+ store depletion involves a cascade of events, which include the oligomerization of STIM1, redistribution and accumulation of STIM1 and Orai1 into over-lapping puncta, and direct interactions between STIM1 and Orai1 through binding of STIM1 at the C- and N-termini of Orai1. However, the alterations that occur in the CRAC channel upon STIM1 binding remain unknown. In this study, we have investigated the role of STIM1 in tuning the biophysical properties of the CRAC channel using mutagenesis and patch-clamp techniques. Our preliminary studies on a non-selective mutant of the CRAC channel reveal that STIM1 binding to Orai not only functions to activate the CRAC channel, but also alters the channel to confer high Ca2+ selectivity and other biophysical properties commonly associated with CRAC channels. Thus, this study provides evidence for a more diverse role for STIM1 in modulating CRAC channel function apart from channel activation.