Orai3-like gating characteristics, in a strongly cooperative manner. In conclusion Orai subtype-specific gating requires a cooperative interplay of all three cytosolic domains.

1000-MiniSymp
Molecular Determinants of CRAC Channel Gating
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Store-operated Ca^{2+} release-activated Ca^{2+} (CRAC) channels regulate numerous cellular functions including transcription, motility, and proliferation in many cell types. The discoveries of Orai1, the CRAC channel pore subunit, and STIM1, the ER Ca^{2+} sensor, have produced rapid progress in our understanding of the molecular features of the CRAC channel pore and the cellular events involved in channel activation. It is now known that following depletion of intracellular Ca^{2+} stores, STIM1 activates CRAC channels by interacting with the cytoplasmic N- and C-terminal domains of Orai1. Orai1 and Orai3 CRAC channels can additionally be activated in a store-independent fashion by the compound 2-APB. However, the molecular and structural mechanisms of STIM1- and 2-APB-mediated gating remain poorly understood. Using a combination of site-directed mutagenesis and cysteine accessibility analysis, we are probing the structural alterations in the pore that occur during STIM1- or 2-APB-mediated activation of Orai1 and Orai3 channels. Our data indicate that the pore of the CRAC channel changes significantly as it transitions from the closed to the open state. Specifically, STIM1 binding causes large modification of the pore architecture, giving rise to features classically associated with CRAC channels such as its narrow pore and high Ca^{2+} selectivity. Thus, in addition to serving as the ER Ca^{2+} sensor and activator of the CRAC channel, STIM1 appears to function as a channel subunit, modifying the structural features of the pore to confer its unique permeation profile in the active state.

1001-MiniSymp
Activation of Orai1 Channels by Mutation of a Conserved Glycine Residue in TM1
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Stim and Orai proteins comprise the molecular machinery of Ca^{2+} release-activated Ca^{2+} (CRAC) channels. In T-lymphocytes, Orai1 is the pore-forming subunit and STIM1 serves as the ER Ca^{2+}-sensor that opens Orai1 following ER Ca^{2+}-store depletion. We are investigating mutations within Orai1 TM1 that alter channel activity in transfected HEK cells. At the extracellular side of TM1, we and others had previously shown that E106 confers Ca^{2+} selectivity on the STIM1-operated Orai1 current. We now show that R91, the site of the R91W mutation that causes severe combined immune deficiency, forms a very narrow part of the conducting pore at the cytosolic side of TM1. Orai1 R91C when co-expressed with STIM1 was activated normally by Ca^{2+} store depletion. However, treatment with diamide, a thiol-oxidizing agent, induced formation of disulfide bonds between R91C residues in adjacent Orai1 subunits. Moreover, diamide rapidly blocked STIM1-operated Ca^{2+} current, and current recovered during treatment with a reducing agent. In the middle of TM1, mutation of a conserved glycine residue to alanine (G98A) prevented STIM1-induced channel activity. Interestingly, mutation to aspartate (G98D) caused constitutive channel activation in a STIM1-independent manner to form a non-selective Ca^{2+}-permeable conductance that was relatively resistant to block by Gd^{3+} (310 nM K_d vs 7 nM in wild-type Orai1). Moreover, the double mutant R91WG98D was also constitutively active, overcoming the normal inhibition of channel activity by tryptophan at the 91 position; and the double mutant R91CG98D was resistant to diamide block. We tentatively propose that R91 forms the gate of the Orai1 channel at the narrow inner mouth of the channel, G98 functions as a gating-hinge, and E106 promotes selective conduction of Ca^{2+}. The channel pore is widened and ion selectivity perturbed by a negative charge at the G98 site.

PLATFORM W: Imaging & Optical Microscopy I

1002-Plat
Circular Scanning Fluorescence Correlation Microscopy on Membranes
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In circular scanning Fluorescence Correlation Spectroscopy (FCS) the measurement volume is scanned in a circle with a sub-micron radius, allowing determination of the diffusion coefficient and concentration without any a priory knowledge of the size of the detection volume. This feature is particularly important in measurements on two-dimensional surfaces, where the volume size, and therefore the quantitative outcome of the experiment, is determined by the relative position of the surface and the objective focus, a parameter difficult to control in practice. We have implemented this technique in a simple instrument based on a 2D piezo scanner, and applied it to studies of molecular diffusion in model systems, supported lipid bilayers and giant unilamellar vesicles, and in living cells. The method is shown to be minimally sensitive to disturbing interferences due to fast kinetics (dye photophysics) and long-time effects (axial membrane motion, depletion due to photobleaching), and yields reliable results even for tilted or fluctuating membranes. The resulting robustness makes this technique particularly suitable to applications in living organisms.

1003-Plat
Probing T-Tubular Electrophysiology by Random Access Two-Photon Microscopy in Cardiac Myocytes
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In cardiac cells many membrane channels are heterogeneously distributed between Surface and T-tubule membranes. Simultaneous recording of membrane potential in the two sarcolemma domains can reveal potential peculiarities of T-tubule electrophysiology. This is not feasible with traditional electrophysiological techniques. Current optical techniques for recording membrane potential can potentially overcome the microelectrode limitation. However, most approaches to the optical recording of membrane potential events lack the spatial and temporal resolution needed for regional assessment of action potential (AP) profile. Here, we developed an ultrafast random access two-photon microscope capable of optically recording fast membrane potential transients in multiple positions of the cell membrane with μm spatial resolution. The random access microscope, in combination with a novel voltage sensitive dye, was used to simultaneously record AP in surface sarcolemma and T-tubules in isolated cardiac myocytes with sub-millisecond time resolution. We found that in myocytes, paced at 0.2 Hz, the AP in the T-tubule has identical amplitude and kinetics as in the surface sarcolemma, indicating that the tight electrical coupling between the two membrane domains prevails over the inhomogeneous distribution of membrane currents. Consistently, in myocytes that had been acutely detubulated by formamide-induced osmotic shock, T-tubule AP was absent, indicating a complete uncoupling from the surface sarcolemma. The electrophysiological properties of t-tubules may be altered in pathological conditions, when detubulation and T-tubule remodelling occur. To mimic a model of pathological detubulation, myocytes were cultured for 24-36 hours, thus obtaining a significant loss and disorganization of the T-tubular network. Membrane staining confirmed the loss and morphological alterations of T-tubules; however, the electrical activity in the remaining remodelled T-tubules was preserved, suggesting that remodelled T-tubules were still coupled to the surface sarcolemma.

1004-Plat
Fluorescence Imaging of Influenza Virus H1N1 mRNA in Living Infected Cells using Single Chromophore FIT-PNA
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Replication of the influenza virus involves, amongst other critical steps, the synthesis of viral mRNA which is used for ribosomal synthesis of viral proteins. Significant efforts have been devoted to the development of methods that allow the imaging of specific mRNA sequences in living cells. In contrast to other approaches, fluorescent oligonucleotide probes enable the analysis of non-modified wild-type targets. For imaging the probes must recognize the target with high specificity and deliver measurable signals that provide high signal-to-background ratios. We have introduced single labelled peptide nucleic acid (PNA) probes, so-called FIT-PNA probes, which contain a single thiazole orange intercalator that serves as artificial fluorescent nucleobase. These probes respond to changes of the local structure in the vicinity of the dye rather than to the more global changes of conformation that are required for fluorescence signalling by the dual labelled molecular beacons. FIT-probes are unique because a single fluorophore provides for both high sensitivity and high target specificity at non-stringent hybridization conditions where both matched and mismatched probe-target complexes coexist. We show that FIT-probes are ideally suited for applications in live cell RNA imaging. We chose mRNA coding for neuraminidase of influenza virus A/PR/8 as a target. FIT-PNA probes are useful to detect the expression of viral mRNA in single living infected cells with high specificity. In particular, FIT-PNA sensitivity was superior to that of a molecular beacon. Our study suggests that FIT-PNAs are attractive tools