The store-operated CRAC channels and the store-independent, arachidonic acid-activated ARC channels represent the founding members of a new family of biophysically similar, highly Ca^{2+}-selective, Ca^{2+} entry channels - the ''Orai channels''. Both of these channels are dependent on STIM1 for their activation, but they differ in the pool of STIM1 responsible. Thus, whereas STIM1 in the ER regulates the CRAC channels on store-depletion, ARC channels are exclusively regulated by the pool of STIM1 that constitutively resides in the PM.
Recent studies have shown that the functional CRAC channel pore is formed by a tetrameric arrangement of Orai1 units. In contrast, a heteropentameric assembly of three Orai1 subunits and two Orai3 subunits forms the functional ARC channel pore (Mignen *et al*. J. Physiol. 587: 4181). Importantly, this inclusion of Orai3 subunits in the channel structure has been shown to play a specific, and unique, role in determining the selectivity of the ARC channels for activation by arachidonic acid. Using an approach based on the generation and expression of various concatenated constructs, we examined the basis for this Orai3-dependent effect on selectivity for arachidonic acid. These studies revealed that, whilst heteropentamers containing only one Orai3 subunit are sensitive to arachidonic acid, *specific selectivity* for activation by this fatty acid is only achieved on inclusion of the second Orai3 subunit in the pentamer. Further studies identified the cytosolic N-terminal domain as the region of the Orai3 molecule that is specifically responsible for this switch in selectivity. Substitution of just this domain into an otherwise complete Orai1 subunit within a concatenated 31111 pentamer is sufficient to change the resulting channel from one that is predominantly store-operated, to one that is essentially exclusively activated by arachidonic acid.

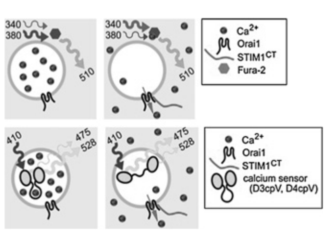
513-Pos
Stim-Dependent and Independent Effects of 2-APB on Orai3 Crac Channels

Megumi Yamashita, Agila Somasundaram, Murali Prakriya.
Northwestern University, Chicago, IL, USA.

The compound 2-aminoethyldiphenyl borate (2-APB) has received widespread attention for its ability to modulate store-operated CRAC channels. 2-APB elicits complex effects in native and ectopic CRAC channels arising from the over-expression of Orai1 (the pore subunit), causing a several-fold enhancement of ICRAC at low concentrations (20 μM). However, recent studies indicate that 2-APB produces strikingly different effects in the Orai3 variant. Here, high 2-APB concentrations activate (rather than inhibit) Orai3 channels. Moreover, the 2-APB activated Orai3 currents differ from store-operated Orai3 (and Orai1) currents in manifesting altered ion selectivity. The multiplicity of 2-APB effects in the different Orai isoforms has confounded efforts to understand its mode of action. Here, we find that 2-APB (50 μM) induces Orai3 current in two kinetically distinct phases: an initial increase in current with no change in ion selectivity is followed by secondary activation of Orai3 channels with altered ion selectivity. Lower concentrations of 2-APB (< 10 μM) potentiated Orai3 currents with no change in ion selectivity, resembling effects seen in Orai1. In contrast to the activation of Orai3 channels by high concentrations of 2-APB, the potentiation by low concentrations of 2-APB was entirely dependent on STIM1. High concentrations of 2-APB also eliminated fast Ca2+-dependent inactivation of Orai3 currents. Collectively, our results indicate that as seen with Orai1 and native CRAC channels, 2-APB causes dual effects on Orai3 channels: low concentrations potentiate Orai3 currents with no change in ion selectivity, whereas high concentrations activate Orai3 currents while also altering ion selectivity and removing fast inactivation. Our results suggest that the complex effects of 2-APB on Orai1 and Orai3 channels share common mechanisms.

514-Pos
Minimal Requirement for Store-Operated Calcium Entry: STIM1 Gates ORAI1 Channels in Vitro

Yubin Zhou[1], Paul Meraner[1], Hyoung T. Kwon[1], Danya Machnes[1], Masatsugu Oh-hora[1], Jochen Zimmer[2], Yun Huang[1], Antonio Stura[1], Anjana Rao[1], Patrick G. Hogan[1].
[1]Immune Diseasea Institute, Harvard Medical School, Boston, MA, USA,
[2]HHMI and Department of Cell Biology, Harvard Medical School, Boston, MA, USA.



Store-operated Ca2+ entry through the plasma membrane CRAC channel in mammalian T cells and mast cells depends on the sensor protein STIM1 and the channel subunit ORAI1. In order to dissect the essential steps in STIM-ORAI signaling in vitro, we have expressed ORAI1 in a sec6-4 strain of the yeast Saccharomyces cerevisiae, which allows isolation of sealed membrane vesicles carrying ORAI1 from the Golgi compartment to the plasma membrane. S cerevisiae itself has no significant reservoir of Ca2+ in the ER, does not possess orthologues of the ER Ca2+-ATPase or IP3 receptor, and has no STIM or ORAI homologues. We show by in vitro Ca2+ flux assays that bacterially-expressed recombinant STIM1 opens wildtype ORAI1 channels, but not channels assembled from the ORAI1 pore mutant E106Q or the ORAI1 immunodeficiency mutant R91W. These experiments demonstrate that the STIM1-ORAI1 interaction is sufficient to gate recombinant human ORAI1 channels in the absence of other proteins of the human ORAI1 channel complex, and set the stage for further biochemical and biophysical dissection of ORAI1 channel gating. (*Y.Z. and P.M. contributed equally to this work.)

515-Pos
Proteomics Analysis of the Drosophila CRAC Channel Complex in the Resting and Active State

Aubin Penna[1], Robyn Kaake[1], Olga Safrina[1], Luette Forrest[1], Andriy V. Yeromin[1], Peter Kaiser[2], Lan Huang[1], Michael D. Cahalan[1].
[1]Department of Physiology & Biophysics, UCI, Irvine, CA, USA,
[2]Department Biological Chemistry, UCI, Irvine, CA, USA.

Recent genome-wide RNAi screens have revealed Stim and Orai as critical components of the Ca^{2+} release-activated Ca^{2+} (CRAC) channel. Upon release of Ca^{2+} from the ER, Stim senses Ca^{2+}depletion, aggregates, relocalizes to ER-plasma membrane (PM) junctions, and interacts with Orai pore-forming subunits in the PM to open the CRAC channel. This signaling cascade is spatially confined, regulated by specific protein-protein interactions between Stim and Orai, and may require additional binding proteins such as regulatory subunits, trafficking proteins, or kinases. We developed an extensive and sensitive proteomics approach to screen for binding partners of Stim and Orai in resting and store-depleted conditions. Histidine-Biotine (HB)-tagged *Drosophila* Stim or Orai proteins were stably expressed in *Drosophila* S2 cells; the HB tag module consisting of a hexahistidine tag (H), a bacterially-derived *in vivo* biotinylation signal peptide (B), and a TEV protease cleavage site (T). HBTH-Orai and Stim-HTBH complexes were purified from resting or Ca^{2+} store-depleted S2 cells lines following two complementary approaches: native purification by high-affinity streptavidin binding and TEV cleavage elution; or, alternatively, *in vivo* chemical cross-linking to freeze both stable and transient interactions in intact cells prior to lysis, followed by tandem-affinity purification (TAP) of the cross-linked protein complexes under fully denaturing conditions. After endoproteolytic digestion and two-dimensional LC, the Stim/Orai interacting proteins were identified by tandem mass spectrometry (MS). By these methods, proteins involved in scaffolding, cytoskeleton dynamics, trafficking, chaperone function, and signaling were identified. In addition to the subunit composition and interacting partners, we also characterized Stim/Orai posttranslational modifications. This work represents the first comprehensive characterization of CRAC channel complex by affinity purification and tandem mass spectrometry and will provide a detailed proteomic profiling of the dynamic protein interaction network in the CRAC channel pathway.

516-Pos
Phosphorylation of STIM1 Underlies Suppression of Store-Operated Calcium Entry During Mitosis

Jeremy T. Smyth, John G. Petranka, Rebecca R. Boyles, Wayne I. DeHaven, Miwako Fukushima, Katina L. Johnson, Jason G. Williams, James W. Putney.
National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA.

When endoplasmic reticulum (ER) Ca^{2+} stores are depleted, Ca^{2+} influx via plasma membrane (PM) Ca^{2+} channels is activated by store-operated Ca^{2+} entry (SOCE). SOCE involves Orai1 Ca^{2+} influx channels and STIM1 ER Ca^{2+} sensors. ER Ca^{2+} depletion induces rearrangement of STIM1 from a diffuse localization throughout the ER membrane into punctate structures near the PM, where it activates Orai1 channels. Interestingly, SOCE is strongly suppressed during mitosis, the only known physiological situation in which SOCE is negatively regulated; however, the mechanisms that underlie SOCE suppression during mitosis are unknown. We found that both endogenous STIM1 and expressed eYFP-tagged STIM1 (eYFP-STIM1) immunoprecipitated from mitotic but not interphase HeLa and HEK293 cells were recognized by the phosphospecific MPM-2 antibody, suggesting mitosis-specific phosphorylation of STIM1. We also found that rearrangement of eYFP-STIM1 into near-PM puncta in response to ER Ca^{2+} depletion was suppressed during mitosis. We therefore hypothesized that STIM1 phosphorylation underlies prevention of STIM1 puncta formation and suppression of SOCE during mitosis. MPM-2 recognizes phospho-serine or threonine followed by proline, and human STIM1 contains 10 occurrences of S/T-P, all downstream of amino acid 482. eYFP-STIM1 truncated at amino acid 482 (482STOP) was not recognized