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

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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

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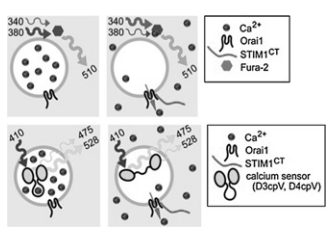
Store-operated Ca2+ entry through the plasma membrane CRAC channel in mammalian T cells and mast cells depends on the sensor protein STIM1 and the channel subunit ORAI1. In order to dissect the essential steps in STIM-ORAI signaling in vitro, we have expressed ORAI1 in a sec6-4 strain of the yeast Saccharomyces 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

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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, cytoskleton 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

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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

by MPM-2 when immunoprecipitated from mitotic cells, suggesting lack of phosphorylation. In support of our hypothesis, non-phosphorylatable 482STOP co-expressed with Orai1 rearranged into near-PM puncta in response to ER Ca^{2+} depletion in mitotic cells, and also significantly rescued mitotic SOCE. A combination of mass spectrometry and site-directed mutagenesis identified S486 and S668 as mitosis-specific phosphorylated residues, and mutation of both to alanine also resulted in partial but significant rescue of SOCE in mitotic cells. Therefore, our data suggest that phosphorylation of S486 and S668 underlies suppression of SOCE during mitosis, although additional phosphorylation sites are likely involved.

517-Pos

Impaired Mitochondria Fail to Ensure Sustained Soce: Possible Mechanism for Decreased Salivary Secretion Under Diabetes

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Xerostomia is a troublesome complication of diabetes mellitus associated with decreased salivation. Previously we showed the diabetes-induced alterations of ACh-mediated $[Ca^{2+}]_{cyt}$ signaling in submandibular salivary gland which provides a major secretion of fluid and electrolytes. Since salivation is initiated by an $InsP_3$-mediated Ca^{2+} release from the ER and subsequently depends on the elevated $[Ca^{2+}]_{cyt}$ maintained by a store-operated Ca^{2+} entry (SOCE), we hypothesized that both processes could be altered under the diabetes contributing to gland dysfunctions. Diabetes was induced by a single i.p. injection of streptozotocin; $[Ca^{2+}]_i$ was measured using fura-2/AM. We found a decrease of the amplitude and deceleration of ACh-induced $[Ca^{2+}]_i$ signals under the diabetes. The increased contribution of mitochondria to the cytosolic calcium clearance in acinar cells was also found under diabetes revealed as: i) an increase in the amount of Ca^{2+}accumulated in mitochondria under basal conditions (by 46%); ii) significantly smaller effect of mitochondrial Ca^{2+} uptake inhibition on the ACh-induced $[Ca^{2+}]_i$ transients in Ca^{2+}-containing extracellular medium (by 69% vs. 29%). Since both SOCE and ER Ca^{2+} refilling are precisely regulated by mitochondria (Kopach et al., 2009), we studied the effectiveness of these processes under diabetes. SOCE induced by short ACh stimulation was increased in diabetic cells (by 70%). Inhibition of mitochondrial Ca^{2+} accumulation equalized SOCE magnitude in control and diabetic cells indicating an increased role of mitochondria to provide positive feedback on SOCE under diabetes. In contrast, during the sustained cells stimulation SOCE was decreased and decelerated under diabetes (~ by 40%) suggesting inability of acinar cells to maintain SOCE under potent agonist stimulation. Concluding, diabetes induces the impairment of intracellular mechanisms responsible for the activation and maintenance of SOCE suppressing mitochondrial Ca^{2+} handling.

518-Pos

Regulation of Vascular Reactivity by Urocortin and Urotensin-II: Role of Store Operated Pathway

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Circulating neuro-hormones, such as Urocortin and Urotensin-II have been demonstrated to critically regulate vascular tone in several arteries. Urocortin was described to induce a strong coronary vasodilatation; beside Urotensin-II was characterized as the most potent mammalian vasoconstrictor identified so far. However, the mechanism of their action is still under debate. The Ca^{2+} independent phospholipase A_2 ($iPLA_2$) dependent activation of store operated Ca^{2+} (SOC) entry have been shown to regulate vascular tone in different arteries. We used vessel myograph, Ca^{2+} imaging, immunocytochemistry and molecular approaches to study the implication of SOC pathway in Urocortin and Urotensin II modulation of rat coronary artery tone.

We observed that Urotensin-II and Urocortin had differential effect on coronary artery. Urocortin induced a potent dose-dependent vasodilatation of agonist-induced coronary contraction. Urocortin activated PKA that inhibited $iPLA_2\beta$ activity and SOC influx in rat SMC. However, Urotensin-II induced a potent vasoconstriction that was sensitive to SOC channel and $iPLA_2$ inhibitors. Urotensin-II produced $iPLA_2\beta$ activation and Ca^{2+} and Mn^{2+} entry in SMC that were inhibited by SOC channels and $iPLA_2\beta$ inhibitors. Interestingly the transfection of SMC with siRNA to Orai1, the pore forming subunit of SOC channels, impaired significantly Urotensin-II induced SOC entry.

These results show that emerging and established transmitter system which can be up- or downregulated in diseases states, regulate differentially the vascular reactivity through the modulation of $iPLA_2\beta$-dependent activation of SOC pathway in coronary artery. This finding is interesting as it gave further information to understand the implication of SOC pathway in physiological and pathological behavior of the coronary artery.

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519-Pos

Calcium Signaling and Prostate Cancer

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Major clinical problem with prostate cancer is the cell's ability to survive and proliferate upon androgen withdrawal. Indeed, deregulated cell proliferation together with the suppression of apoptosis provides the condition for abnormal tissue growth.

Alterations in Ca^{2+} homeostasis have been described to increase proliferation, to induce differentiation or apoptosis. During the last years it has emerged that several members of the TRP family could play an important role in prostate carcinogenesis and even more, some of them have been suggested as a prognostic markers for PCa especially useful in the differential diagnosis.

We were particularly interested by TRPM8 channels since TRPM8 is a target gene of the androgen receptor and its expression strongly increases in prostate cancer. Recent evidence we have obtained indicate that TRPM8 may be expressed not just in the plasma membrane, but also in the endoplasmic reticulum (ER) membrane where TRPM8 may operate as an ER Ca^{2+} release channel. The ''preferred'' TRPM8 localization depends on epithelial cell phenotype (differentiated apical cells vs. non-differentiated basal cells) and on androgen status (androgen-dependent vs. hormone refractory. New results on the differential physiological role of TRPM8 isoforms in prostate cancer cells will be presented.

520-Pos

Frequent Calcium Oscillations Lead to NFAT Activation in Human Immature Dendritic Cells

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Spontaneous Ca2+ oscillations have been reported in certain types of excitable and non-excitable cells. However, the precise molecular mechanism underlying these events and their biological role(s) remain unclear. In the present study we demonstrate for the first time that spontaneous Ca2+ oscillations occur in immature human monocyte-derived dendritic cells and that upon receiving maturation signals via TLRs, the cells lose the high frequency Ca2+ oscillations. We investigated the mechanism and role of the Ca2+ oscillations in immature dendritic cells and found that the inositol-1,4,5-trisphosphate receptor is essential, since oscillations were blocked by pre-treatment of cells with the inositol-1,4,5-trisphosphate receptor antagonist Xestospongin C and 2-APB. A component of the Ca2+ signal is also due to influx from the extracellular environment and may be involved in refilling the intracellular Ca2+ stores. As to their biological role, our results indicate that they are intimately linked to the ''immature'' phenotype and are associated with the translocation of the transcription factor NFAT into the nucleus. In fact, blocking the Ca2+ oscillations with 2-APB or treating the cells with LPS, leading then to undergo maturation, caused NFAT to remain in the cytoplasm. The results presented in this report provide novel insights into the physiology of immature dendritic cells and into the signaling process(es) controlling their maturation.

521-Pos

A Reduction of Spontaneous Beating Rate of Adult Rabbit Pacemaker Cells in Culture is Reversed by RGS2 Overexpression, Gi Inhibition or β-Ar Stimulation

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Genetic manipulation of signaling proteins is an important tool to study signaling mechanisms. While rabbit sinoatrial node cells (SANC) are an excellent model for the study of autonomic signaling, genetic manipulation of freshly isolated rabbit SANC (f-SANC) is not possible. Here we report important characteristics of a cultured rabbit SANC model (c-SANC) that is suitable for manipulation of gene expression. C-SANC generate regular and rhythmic APs at 34 ± 0.5^{o}C, and beat spontaneously at a lower rate (1.35 ± 0.02Hz, n=803) than f-SANC (2.79 ± 0.04Hz, n=203 $p<0.001$). The durations of AP and Ca^{2+}-transient are prolonged in c-SANC. Spontaneous Local Ca^{2+}-Releases (LCR) beneath cell membrane during diastolic depolarization have prolonged period that is highly correlated with c-SANC's reduced spontaneous beating rate.