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WGA, vinculin and β-tubulin. Additionally, no stretch-activated current was detected when stretching isolated cardiomyocytes to approximately 10% of their resting length. Taken together, our data indicate that TRPC1 and TRPC6 are not located in the sarcolemma, but in the membrane of the SR adjacent to the Z-disks in rabbit ventricular myocytes. We suggest that a potential role of TRPC1 and TRPC6 channels is in stretch-dependent calcium leak from the SR and/or counter current balancing calcium release from the SR through ryanodine receptors.

3816-Pos Board B544

Single Molecule Optical Recordings of TRPV1 Mobility and Activity

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Inflammatory signals increase the excitability of TRPV1-expressing nociceptors, at least in part, by increasing the number TRPV1 channels in the plasma membrane (PM) of nociceptors. However, the dynamics of TRPV1 in the PM have not been studied.

Combining TIRF microscopy with whole-cell patch clamp of isolated mouse sensory neurons and HEK293T/17 cells allowed us to image both the localization of single TRPV1 molecules within cells (TRPV1-eGFP) and their activity (capsaicin-activated fluorescent "sparklets" at sites of Ca^{2+} influx). Our single-molecule approach revealed the following: (1) TRPV1 channels in isolated sensory neurons and in cultured cells exhibit high lateral mobility; (2) sparklet activity elicited by capsaicin in isolated sensory neurons reveal that TRPV1 is free to move laterally in the plasma membrane while conducting Ca^{2+} into the cytosol; (3) Two-state sparklet fluorescence and photobleaching analysis indicate that TRPV1 activity is not contingent on the formation of higher order structures; (4) the lateral mobility of TRPV1 in the plasma membrane decreased as a function of open duration; and (5) after addition of capsaicin, influx of Ca^{2+} but not Na^{+} slowed the ensemble mobility of TRPV1.

Although the mechanism by which TRPV1 activity and TRPV1 mobility are coupled and the role of mobility changes in cell signaling remain to be determined, our data demonstrate the power of single-molecule measurements to reveal aspects of signaling not observable in macroscopic experiments. Our data suggest that changes to the dynamic localization of TRPV1 by its activation may constitute a new form of regulation.

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TRPC3 Modulates Association of Orai1 with Immunophilin FKBP12 and Orai-Mediated Ca^{2+}-Transcription Coupling in Mast Cells

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Various Ca^{2+}-permeable channels were demonstrated to associate with immunophilins (FKBP12) as well as calcineurin in a wide range of cellular systems. A possible task of such signalosomes may be efficient translation of local calcium signals into control of gene expression. In mast cells, transcriptionally relevant Ca^{2+}-entry is mainly mediated by store-operated Orai channels or by the canonical transient receptor potential channel (TRPC) family. However, as yet little is known as to how these Ca^{2+}-permeable channels interact with FKBP12 and calcineurin and how they contribute to NFAT signaling in mast cells.

We investigated the role of TRPC3 and Orai1 channels in NFAT signaling of RBL-2H3 mast cells using electrophysiology in combination with TIRF/FRET fluorescence microscopy and heterologous expression of mutant channel proteins as well as genetic knockdown by siRNA. Ca^{2+} entry via Orai1 but not TRPC3 was required for store depletion-induced NFAT activation, as indicated by the lack of effect observed after expression of a dominant negative, pore-dead TRPC3 mutant (TRPC3E630K) as well as siRNA knock-down of TRPC3. Nonetheless, overexpression of the STIM1 binding deficient mutant TRPC3D698/699K or of FKBP12 binding deficient TRPC3P704Q substantially inhibited Orai1-dependent NFAT translocation in RBL-2H3 cells. Association of TRPC3 with FKBP12 was demonstrated by TIRF/FRET microscopy and the FRET signal was strongly reduced by replacing TRPC3 by TRPC3P704Q. Interestingly, association of Orai1 into FKBP12-containing signalosomes was similarly observed in TIRF/FRET experiments, and overexpression of TRPC3 facilitated the association of Orai1 with FKBP12 as well as calcineurin. Moreover, these interactions were reduced by overexpression of TRPC3P704Q.

From our data we conclude that TRPC3 is a structural element of an immunophilin comprising protein complex in mast cells, thereby facilitating the interaction of Orai1 with FKBP12/calcineurin and NFAT signaling.

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Ca^{2+} Facilitates TRPC4 Activation by Gi/O Signaling in Both Calmodulin Dependent and Independent Manner

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TRPC4 is a member of the Canonical Transient Receptor Potential family of non-selective ion channels and is regulated by a set of intracellular and extracellular factors including G protein coupled receptor activation, calcium and membrane phospholipids. Sodium (Na^{+}) and calcium (Ca^{2+}) influx through the active TRPC4 channel elicits membrane depolarization and intracellular Ca^{2+} signaling in neurons, vascular endothelium and smooth muscle cells. Uniquely, TRPC4 is maximally activated only when two separate G protein pathways, $G_{q/11}$ and $G_{i/o}$, are co-stimulated, making it a coincidence detector of $G_{q/11}$- and $G_{i/o}$-coupled receptor co-activation. This function may be important for coordinating the actions of sympathetic (e.g. adrenergic) and parasympathetic (e.g. cholinergic) innervations. While a direct interaction between $G_{i/o\text{-alpha}}$ subunits and the cytoplasmic C-terminal domain of TRPC4 has been suggested to underlie the activation mechanism by $G_{i/o}$ proteins, the PLC-IP_3-IP_3R pathway is thought to be critical for the action of $G_{q/11}$. Using electrophysiological recordings of HEK293 cells heterologously expressing mouse TRPC4-beta, we examined the interdependence of $G_{i/o}$ and $G_{q/11}$ pathways on TRPC4 activation. We show that whereas $G_{i/o}$ (but not $G_{q/11}$) stimulation is necessary, it does not fully activate TRPC4 unless accompanied by a simultaneous rise in intracellular free Ca^{2+}, which may or may not require activation of the $G_{q/11}$ pathway. Paradoxically, inhibiting calmodulin also facilitated TRPC4 activation by $G_{i/o}$ signaling, but not to the full extent as that facilitated by increasing intracellular Ca^{2+} levels, indicating an inhibitory role played by calmodulin on TRPC4 activation and a calmodulin-independent positive regulation by Ca^{2+}. Our findings indicate that TRPC4 is a coincidence sensor of (A) $G_{i/o}$ stimulation and (B) intracellular $[Ca^{2+}]$ rise that can be triggered by receptor-PLC pathways, lysosomal Ca^{2+} release, ER Ca^{2+} store release and plasma membrane Ca^{2+} influx mechanisms.

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Exploring the Architecture of the Outer Pore of the TRPV1 Channel with Double-Knot Toxin

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The TRPV1 channel is a non-selective homotetrameric cation channel that acts in nociceptors as a sensor for external noxious chemicals and physical stimuli. It is also modulated by many cell-signaling molecules whose concentrations increase during inflammation and tissue damage. As a consequence, its function has been associated with inflammatory hyperalgesia and neuropatic pain. At present, little structural or mechanistic information is available for this protein. However, the pore domain is thought to play a fundamental role in channel function, since modulating signals are expected to converge on the pore to gate ion permeation. There is also growing evidence to suggest that the pore is the site of action of protons, possibly temperature, and the double ICK knot tarantula toxin (DkTx). DkTx, or its isolated single knots, have been proposed to bind to the external pore to promote activation. Here, we set out to study DkTx-TRPV1 interactions to gain structural information on the pore domain. By using a concatenated TRPV1 channel tetramer, in which toxin-activation was disrupted in specific subunits, we found that the binding of a single toxin knot is sufficient to promote channel activation. We also found that DkTx activates channels in which toxin-activation has been disrupted in either adjacent or opposite subunits, indicating that the knots of DkTx can arrange differently to activate the TRPV1 channel. Finally, we found that a hexa-histidine tag attached to the N-terminus of the toxin acts a voltage- and pH-dependent blocker. We are currently using variants of DkTx, in combination with concatenated TRPV1 channels, to delineate the toxin binding site on the external surface of the channel relative to the central pore and to probe agonist-dependent conformational changes in the pore.