2336-Pos Board B355

Selective Regulation of Phosphoinositide Levels in Nociceptive Neurons Underlie Differential Regulation of TRPV1 Channels

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Transient Receptor Potential Vanilloid 1 (TRPV1) ion channels are regulated in a complex manner. Activation of G-protein coupled receptors (GPCR) by inflammatory mediators sensitizes the channels to moderate stimuli, whereas prolonged pharmacological activation of TRPV1 leads to diminished activity over time (desensitization). Paradoxically, both conditions entail activation of phospholipase C (PLC) enzymes. We found that in sensory neurons activation of GPCRs by bradykinin leads to a moderate decrease in phosphatidylinositol 4,5 bisphosphate [$PI(4,5)P_2$], but no sustained change in the levels of its precursor PI(4)P. Preventing the decrease of $PI(4,5)P_2$ inhibited bradykinin-induced sensitization, while selectively decreasing $PI(4,5)P_2$ potentiated sensitization induced by submaximal activation of protein kinase C (PKC). Disruption of the tubulin cytoskeleton with colchicine mimicked the effect of decreasing $PI(4,5)P_2$ levels. On the other hand maximal stimulation with capsaicin lead to a robust decrease of both $PI(4,5)P_2$ and its precursor PI(4)P in sensory neurons. Capsaicin-induced desensitization of TRPV1 currents was significantly reduced by intracellular dialysis of either $PI(4,5)P_2$ or PI(4)P. Combined reduction of $PI(4,5)P_2$ and PI(4)P without the activation of PLC reduced TRPV1 activity. We propose a comprehensive model in which selective changes in phosphoinositide levels by distinct PLC isoforms mediate the differential regulation of TRPV1 by GPCR activation and by Ca^{2+} influx.

2337-Pos Board B356

The Roles Calmodulin and $PI(4,5)P_2$ in Ca^{2+}-Induced Inactivation of TRPV6 Channels

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The epithelial Ca^{2+} channel Transient Receptor Potential Vanilloid 6 (TRPV6) undergoes Ca^{2+}-induced inactivation. To dissect the roles of individual signaling pathways in this phenomenon, we studied the effects of Ca^{2+}, CaM and phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] in excised patches. The activity of TRPV6 decreased after excision, (run down) and channels could be re-activated with either $PI(4,5)P_2$ or MgATP, which supplies substrate for endogenous lipid kinases to synthesize $PI(4,5)P_2$. Ca^{2+}-CaM inhibited the channel at physiologically relevant concentrations both when endogenous $PI(4,5)P_2$ was used to maintain channel activity, and when exogenous $PI(4,5)P_2$ was applied. Ca^{2+} alone also inhibited TRPV6 at high concentrations (IC_{50} ~20 μM). Biochemical binding experiments showed that CaM binds to TRPV6 via a distal C-terminal binding site. A double mutation in this CaM binding site of TRPV6 (W695A-R699E) essentially eliminated inhibition by CaM in excised patches. In whole-cell patch clamp experiments, this mutation reduced, but did not eliminate Ca^{2+}-induced inactivation. Providing excess $PI(4,5)P_2$ reduced the inhibition by CaM in excised patches and in planar lipid bilayers, but $PI(4,5)P_2$ did not inhibit binding of the C-terminus of the channel to CaM. Overall, our data show a complex interplay between CaM and $PI(4,5)P_2$, and that Ca^{2+}, CaM and the depletion of $PI(4,5)P_2$ all contribute to inactivation of TRPV6.

2338-Pos Board B357

Regulation of Calcium Influx and Signaling Pathway in Cancer Cells via TRPV6-Numb1 Interaction

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TRPV6 is overexpressed in some cancers and exhibits oncogenic potential, but its exact mechanism is still poorly understood. The Numb protein is a cell fate determinant that functions in endocytosis and as a tumor suppressor via the stabilization of p53. Here, we showed a novel function of Numb1, which negatively regulates TRPV6 activity. The expression of Numb1 decreased cytosolic Ca^{2+} concentrations in TRPV6-transfected HEK293 cells. When all the isoforms of Numb were depleted using siRNA in a TRPV6 stable cell line, the levels of cytosolic Ca^{2+} increased. We observed an interaction between Numb1 and TRPV6 using co-immunoprecipitation. We confirmed this interaction using Fluorescence Resolution Energy Transfer (FRET). We identified the TRPV6 and Numb1 binding site using TRPV6 C-terminal truncation mutants and Numb1 deletion mutants. The binding site in TRPV6 was an aspartic acid at amino acid residue 716, and that binding site in Numb1 was arginine at amino acid residue 434. A Numb1 mutant, lacking TRPV6 binding activity, failed to inhibit TRPV6 activity. Every isoform of Numb knockdown, using an siRNA-based approach in MCF-7 breast cancer cells, not only showed enhanced TRPV6 expression but also both the cytosolic Ca^{2+} concentration and cell proliferation were increased. The down-regulated expression of TRPV6 using siRNA increased Numb protein expression; however, the cytosolic influx of Ca^{2+} and proliferation of the cell were decreased. To examine downstream signaling during Ca^{2+} influx, we performed western blotting analysis on TRPV6 upregulated cancer cells (MCF-7, PC-3). Taken together, these results demonstrated that Numb1 interacts with TRPV6 through charged residues and inhibits its activity via the regulation of protein expression. Moreover, we provided evidence for a Ca^{2+}-regulated cancer cell signaling pathway and that the Ca^{2+} channel is a target of cancer cells.

2339-Pos Board B358

TRPC 1 acts as a Negative Regulator for TRPV6 Mediated Ca^{2+} Influx

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TRP proteins mostly assemble to homomeric channels but can also heteromerize, preferentially within their subfamilies. The TRPC1 protein is the most versatile member and forms various TRPC channel combinations but also unique channels with the distantly related TRPP2 and TRPV4. We show here a novel cross-family interaction between TRPC1 and TRPV6, a Ca^{2+} selective member of the vanilloid TRP subfamily. TRPV6 exhibited substantial co-localization and in vivo interaction with TRPC1 in HEK293 cells, however, no interaction was observed with TRPC3, TRPC4 or TRPC5. Ca^{2+} and Na+ currents of TRPV6 over-expressing HEK293 cells are significantly reduced by co-expression of TRPC1, correlating with a dramatically suppressed plasma membrane targeting of TRPV6. In line with their intracellular retention, remaining currents of TRPC1 and TRPV6 co-expression resemble in current-voltage relationship that of TRPV6. Studying the N-terminal ankyrin like repeat domain, structurally similar in the two proteins, we have found that these cytosolic segments were sufficient to mediate a direct heteromeric interaction. Moreover, the inhibitory role of TRPC1 on TRPV6 influx was also maintained by expression of only its N-terminal ankyrin-like repeat domain. Our experiments provide evidence for a functional interaction of TRPC1 with TRPV6 that negatively regulates Ca^{2+} influx in HEK293 cells.

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2340-Pos Board B359

Role of C-Terminus in the Function of the Yvc1p Saccharomyces Cerevisiae TRP Channel

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Yvc1p is a TRP channel found on the vacuolar membrane of *Saccharomyces cerevisiae*. Yvc1p plays a key role in Ca^{2+} homeostasis and can be activated by hyperosmotic shock[1]. The transmembrane topology of TRP channels is very similar to voltage-dependent K^+ channels: there are six transmembrane domains and the N- and C-termini are generally depicted as being cytoplasmic. Yvc1p is unusual in that its N- and C-termini are much lengthier than most related TRP channels. In this study, we have focused on understanding the contribution of the C-terminus to the activity of Yvc1p.

The C- terminus contains ~210 amino acid residues. within this segment, there is only one putative domain: a DDDD motif that is thought to bind calcium[2]. We are using two different functional assays to examine a panel of C-terminal deletion constructs. We used an *in vivo* luminescence assay to monitor channel activity induced by hyperosmotic shock. In this assay, luminescence is generated when Ca^{2+}, released from the vacuole by Yvc1p, binds to cytoplasmic aequorin. We find that aequorin luminescence is adversely affected by deletions of more than the last 12 residues. The second functional assay examines a Yvc1p-dependent phenotype observed during growth on agar plates: Yvc1p overexpression causes diminished growth in high [Ca^{2+}][2]. With this assay, constructs truncated in the region of 578 and 633 affect growth in high [Ca^{2+}], but also in the control conditions of low salt and high [Mg^{2+}] as well, suggesting perturbations of this region may have non-specific consequences for channel function. Ongoing experiments examine how the level and localization of Yvc1p is affected by truncations of the C-terminus.

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